IMPROVED GRAIN QUALITY THROUGH ALTERED EXPRESSION OF SEED PROTEINS

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FIELD OF THE INVENTION

The invention relates to the field of plant molecular biology and the use of genetic modification to improve the quality of crop plants, more particularly to methods for improving the nutritional value of grain and the efficiency of grain processing.

DETAILED DESCRIPTION OF THE INVENTION

The present invention is directed to compositions and methods for altering the levels of seed proteins in plant seed, particularly reducing the levels of gamma-zein proteins in maize and the levels of gamma-kafarin in sorghum. Modification of seed protein composition causes changes in the physical and/or chemical properties of the grain.

The invention is directed to the alteration of protein levels in plant seed, resulting in grain with increased digestibility/nutrient availability, improved amino acid composition/nutrient value, increased response to feed processing, improved silage quality, increased efficiency of wet or dry milling, and decreased allergenicity and/or toxicity. The claimed sequences encode proteins preferentially expressed during seed development.

As used herein, "grain" means the mature seed produced by commercial growers for purposes other than growing or reproducing the species.

Additional uses of the invention include increasing seed hardness, decreasing seed caloric value for use in diet foods and other food for human use, pet food, increasing the antioxidant properties of seed, and taking advantage of the metal chelating properties of the legumin 1 protein of the invention to purify other polypeptides of interest.

Compositions of the invention comprise sequences encoding maize seed proteins and variants and fragments thereof. Methods of the invention involve the use of transgenic expression, antisense suppression, co-suppression, mutagenesis including transposon tagging, and biosynthetic competition to manipulate, in plants and plant seeds and grains, the expression of seed proteins, including, but not limited to, those encoded by the sequences disclosed herein. Transgenic plants producing seeds and grain with altered seed protein content are also provided. The modified seed and grain of the invention may be obtained by breeding crosses with the transgenic plants, such as by marker assisted selected breeding. The 50 kD gamma-zein of the instant invention maps to chromosome 7, bin 7.03, the 18 kD alpha-globulin to chromosome 6, bin 6.05, and the 50 kD legumin 1 to chromosome 6, Bin 6.01. This information enables one of skill in the art to employ these map locations to generate improved maize lines with altered seed protein levels.

It is recognized that while the invention is exemplified by the modulation of expression of selective sequences in maize, similar methods can be used to modulate the levels of seed proteins in other plants, particularly other cereals such as sorghum. In this manner, the sequences of the invention can be used to identify and isolate similar sequences in other plants based on sequence homology or sequence identity. Alternatively, where the maize sequences share sufficient homology to modulate expression of the native genes, such as in sorghum, the maize sequences may be used to modulate expression in sorghum. For a review of sorghum seed proteins including gamma-kafarin see Leite *et al.*, *The Prolamins of Sorghum, Coix and Millets.*, In: Shewry and Casey (eds.) (1999) *Seed Proteins*, 141-157, Academic Publishers, Dordrecht.

In particular, the present invention provides isolated nucleic acid molecules comprising a nucleotide sequence encoding a maize protein, designated herein as

the 50 kD gamma-zein, having the amino acid sequence shown in SEQ ID NO:2, or the nucleotide sequence encoding the DNA sequence deposited in a bacterial host as Patent Deposit No. PTA-2272. Further provided is a polypeptide having an amino acid sequence encoded by the nucleic acid molecules described herein, for example that set forth in SEQ ID NO:1, and deposited in a bacterial host as Patent Deposit No. PTA-2272, and fragments and variants thereof.

The present invention also provides isolated nucleic acid molecules comprising a nucleotide sequence encoding a maize protein, herein designated as the 18 kD alpha-globulin, having the amino acid sequence shown in SEQ ID NO:4, or the nucleotide sequence encoding the DNA sequence deposited in a bacterial host as Patent Deposit No. PTA-2274. Further provided is a polypeptide having an amino acid sequence encoded by the nucleic acid molecules described herein, for example that set forth in SEQ ID NO:3, and deposited in a bacterial host as Patent Deposit No. PTA-2274, and fragments and variants thereof.

The present invention also provides isolated nucleic acid molecules comprising a nucleotide sequence encoding a maize protein, herein designated as the 50 kD legumin 1 prolamin, having the amino acid sequence shown in SEQ ID NO:6, or the nucleotide sequence encoding the DNA sequence deposited in a bacterial host as Patent Deposit No. PTA-2273. Further provided is a polypeptide having an amino acid sequence encoded by the nucleic acid molecules described herein, for example that set forth in SEQ ID NO:5, and deposited in a bacterial host as Patent Deposit No. PTA-2273, and fragments and variants thereof.

A plasmid containing the nucleotide sequence encoding the 50 kD gamma-zein protein was deposited with the Patent Depository of the American Type Culture Collection (ATCC), Manassas, Virginia, on July 26, 2000 and assigned Patent Deposit No. PTA-2272. A plasmid containing the nucleotide sequence encoding the 18 kD alpha-globulin protein was deposited with the Patent Depository of the American Type Culture Collection (ATCC), Manassas, Virginia, on July 26, 2000 and assigned Patent Deposit No. PTA-2274. A plasmid containing the nucleotide sequence encoding the 50 kD legumin 1 protein was deposited with the Patent Depository of the American Type Culture Collection (ATCC), Manassas, Virginia, on

July 26, 2000 and assigned Patent Deposit No. PTA-2273. These deposits will be maintained under the terms of the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purposes of Patent Procedure. These deposits were made merely as a convenience for those of skill in the art and are not an admission that a deposit is required under 35 U.S.C. §112.

A comparison of the amino acid content of cereal grains shows that 18 kD alpha-globulin is an excellent source of tryptophan and methionine for amino acid balance in all cereals and that 50 kD legumin 1 prolamin is an excellent source of methionine for all cereals and a good source of lysine and tryptophan for the amino acid balance of most cereals.

The invention encompasses isolated or substantially purified nucleic acid or protein compositions. An "isolated" or "purified" nucleic acid molecule or protein, or biologically active portion thereof, is substantially or essentially free from components that normally accompany or interact with the nucleic acid molecule or protein as found in its naturally occurring environment. Thus, an isolated or purified nucleic acid molecule or protein is substantially free of other cellular material, or culture medium when produced by recombinant techniques, or substantially free of chemical precursors or other chemicals when chemically synthesized. Preferably, an "isolated" nucleic acid is free of sequences (preferably protein encoding sequences) that naturally flank the nucleic acid (i.e., sequences located at the 5' and 3' ends of the nucleic acid) in the genomic DNA of the organism from which the nucleic acid is derived. For example, in various embodiments, the isolated nucleic acid molecule can contain less than about 5 kb, 4 kb, 3 kb, 2 kb, 1 kb, 0.5 kb, or 0.1 kb of nucleotide sequences that naturally flank the nucleic acid molecule in genomic DNA of the cell from which the nucleic acid is derived. A protein that is substantially free of cellular material includes preparations of protein having less than about 30%, 20%, 10%, 5%, (by dry weight) of contaminating protein. When the protein of the invention or biologically active portion thereof is recombinantly produced, preferably culture medium represents less than about 30%, 20%, 10%, or 5% (by dry weight) of chemical precursors or non-protein-of-interest chemicals.

Fragments and variants of the disclosed nucleotide sequences and proteins encoded thereby are also encompassed by the present invention. By "fragment" is intended a portion of the nucleotide sequence or a portion of the amino acid sequence and hence protein encoded thereby. Fragments of a nucleotide sequence may encode protein fragments that retain the biological activity of the native 50 kD gamma-zein, the 18 kD alpha-globulin, or the 50 kD legumin 1 proteins. Alternatively, fragments of a nucleotide sequence that are useful as hybridization probes generally do not encode fragment proteins retaining biological activity. Thus, fragments of a nucleotide sequence may range from at least about 40 nucleotides, about 50 nucleotides, about 100 nucleotides, and up to the full-length nucleotide sequence encoding the native 50 kD gamma-zein, the 18 kD alpha-globulin, or the 50 kD legumin 1 protein of the invention.

Fragments of the maize nucleotide sequences of the invention (SEQ ID NO:1, SEQ ID NO:3, or SEQ ID NO:5) that encode a biologically active portion of the 50 kD gamma-zein protein, the 18 kD alpha-globulin protein, or the 50 kD legumin 1 protein of the invention, respectively, will encode at least 15, 25, 30, 50, 100, 150, or 200 contiguous amino acids, or up to the total number of amino acids present in the full-length 50 kD gamma-zein protein, the 18 kD alpha-globulin protein, or the 50 kD legumin 1 protein of the invention (for example, 295 amino acids for SEQ ID NO:1; 206 amino acids for SEQ ID NO:3; and 483 amino acids for SEQ ID NO:5). Fragments of SEQ ID NO:1, SEQ ID NO:3, and SEQ ID NO:5 that are useful as hybridization probes or PCR primers need not encode a biologically active portion of a prolamin protein.

Thus, a fragment of SEQ ID NO:1, SEQ ID NO:3, or SEQ ID NO:5 may encode a biologically active portion of a prolamin protein, or it may be a fragment that can be used as a hybridization probe or PCR primer using methods disclosed below. A biologically active portion of the 50 kD gamma-zein protein, the 18 kD alpha-globulin protein, or the 50 kD legumin 1 protein of the invention can be prepared by isolating a portion of the disclosed nucleotide sequence that codes for a portion of the 50 kD gamma-zein protein, the 18 kD alpha-globulin protein, or the 50 kD legumin 1 protein (e.g., by recombinant expression *in vitro*), and assessing the activity of the

encoded portion of the prolamin protein. Nucleic acid molecules that are fragments of SEQ ID NO:1 comprise at least 40, 50, 75, 100, 150, 200, 250, 300, 350, 400, 450, 500, 550, 600, 650, 700, 800, 900, 1000, or 1100 nucleotides, or up to the number of nucleotides present in the full-length gamma-zein cDNA (for example, 1129 nucleotides for SEQ ID NO:1). Nucleic acid molecules that are fragments of SEQ ID NO:3 comprise at least 40, 50, 75, 100, 150, 200, 250, 300, 350, 400, 450, 500, 550, 600, 650, 700, 800, or 900 nucleotides, or up to the number of nucleotides present in the full-length alpha-globulin cDNA (for example, 950 nucleotides for SEQ ID NO:3). Nucleic acid molecules that are fragments of SEQ ID NO:5 comprise at least 40, 50, 75, 100, 150, 200, 250, 300, 350, 400, 450, 500, 550, 600, 650, 700, 800, 1000, 1200, 1400, or 1600 nucleotides, or up to the number of nucleotides present in the full-length legumin 1 prolamin cDNA (for example, 1679 nucleotides for SEQ ID NO:5).

By "variants" is intended substantially similar sequences. For nucleotide sequences, conservative variants include those sequences that, because of the degeneracy of the genetic code, encode the amino acid sequence of 50 kD gamma-zein protein, the 18 kD alpha-globulin protein, or the 50 kD legumin 1 protein of the invention. Naturally occurring allelic variants such as these can be identified with the use of well-known molecular biology techniques, as, for example, with polymerase chain reaction (PCR) and hybridization techniques as outlined below. Variant nucleotide sequences also include synthetically derived nucleotide sequences, such as those generated, for example, by using site-directed mutagenesis but which still encode a 50 kD gamma-zein protein, an 18 kD alpha-globulin protein, or an 50 kD legumin 1 protein. Generally, variants of a particular nucleotide sequence of the invention will have at least about 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% or more sequence identity to that particular nucleotide sequence as determined by sequence alignment programs described elsewhere herein using default parameters.

By "variant" protein is intended a protein derived from the native protein by deletion (so-called truncation) or addition of one or more amino acids to the N-terminal and/or C-terminal end of the native protein; deletion or addition of one or

more amino acids at one or more sites in the native protein; or substitution of one or more amino acids at one or more sites in the native protein. Variant proteins encompassed by the present invention are biologically active, that is they continue to possess the activity of the native prolamin proteins of the invention as described herein. Such variants may result from, for example, genetic polymorphism or from human manipulation. Biologically active variants of the native 50 kD gamma-zein protein, the 18 kD alpha-globulin protein, or the 50 kD legumin 1 protein of the invention will have at least about 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% or more sequence identity to the amino acid sequence for the native protein as determined by sequence alignment programs described elsewhere herein using default parameters. A biologically active variant of a protein of the invention may differ from that protein by as few as 1-15 amino acid residues, as few as 1-10, such as 6-10, as few as 5, as few as 4, 3, 2, or even 1 amino acid residue.

The proteins of the invention may be altered in various ways including amino acid substitutions, deletions, truncations, and insertions. Methods for such manipulations are generally known in the art. For example, amino acid sequence variants of the 50 kD gamma-zein protein, the 18 kD alpha-globulin protein, or the 50 kD legumin 1 protein can be prepared by mutations in the DNA. Methods for mutagenesis and nucleotide sequence alterations are well known in the art. See, for example, Kunkel (1985) *Proc. Natl. Acad. Sci. USA 82*:488-492; Kunkel *et al.* (1987) *Methods in Enzymol. 154*:367-382; US Patent No. 4,873,192; Walker and Gaastra, eds. (1983) *Techniques in Molecular Biology* (MacMillan Publishing Company, New York) and the references cited therein. Guidance as to appropriate amino acid substitutions that do not affect biological activity of the protein of interest may be found in the model of Dayhoff *et al.* (1978) *Atlas of Protein Sequence and Structure* (Natl. Biomed. Res. Found., Washington, D.C.), herein incorporated by reference.

Thus, the genes and nucleotide sequences of the invention include both the naturally occurring sequences as well as mutant forms. Likewise, the proteins of the invention encompass both naturally occurring variant proteins as well as variations and modified forms thereof. Such variants will continue to possess the native seed

protein activity. Obviously, the mutations that will be made in the DNA encoding the variant must not place the sequence out of reading frame and preferably will not create complementary regions that could produce secondary mRNA structure. See EP Patent Application Publication No. 75,444.

Variant nucleotide sequences and proteins also encompass sequences and proteins derived from a mutagenic and recombinogenic procedure such as DNA shuffling. With such a procedure, one or more different gamma-zein, alpha-globulin, or legumin protein coding sequences can be manipulated to create a new gamma-zein, alpha-globulin, or legumin protein possessing the desired properties. In this manner, libraries of recombinant polynucleotides are generated from a population of related sequence polynucleotides comprising sequence regions that have substantial sequence identity and can be homologously recombined *in vitro* or *in vivo*. For example, using this approach, sequence motifs encoding a domain of interest may be shuffled between 50 kD gamma-zein coding sequence, the 18 kD alpha-globulin coding sequence, or the 50 kD legumin 1 prolamin coding sequence of the invention and other known gene coding sequences to obtain a new coding sequence for a protein with an improved property of interest. Strategies for such DNA shuffling are known in the art. See, for example, Stemmer (1994) *Proc. Natl. Acad. Sci. USA 91*:10747-10751; Stemmer (1994) *Nature 370*:389-391; Crameri *et al.* (1997) *Nature Biotech. 15*:436-438; Moore *et al.* (1997) *J. Mol. Biol. 272*:336-347; Zhang *et al. (1997) Proc. Natl. Acad. Sci. USA 94*:4504-4509; Crameri *et al.* (1998) *Nature 391*:288-291; and U.S. Patent Nos. 5,605,793 and 5,837,458.

The nucleotide sequence of the invention can be used to isolate corresponding sequences from other plants. In this manner, methods such as PCR, hybridization, and the like can be used to identify such sequences based on their sequence homology to the sequence set forth herein. Sequences isolated based on their sequence identity to the entire 50 kD gamma-zein, 18 kD alpha-globulin, or 50 kD legumin 1 sequences set forth herein or to fragments thereof are encompassed by the present invention. Such sequences include sequences that are orthologs of the disclosed sequences. By "orthologs" is intended genes derived from a common ancestral gene and which are found in different species as a result of

speciation. Genes found in different species are considered orthologs when their nucleotide sequences and/or their encoded protein sequences share substantial identity as defined elsewhere herein. Functions of orthologs are often highly conserved among species.

In a PCR approach, oligonucleotide primers can be designed for use in PCR reactions to amplify corresponding DNA sequences from cDNA or genomic DNA extracted from any plant of interest. Methods for designing PCR primers and PCR cloning are generally known in the art and are disclosed in Sambrook *et al.* (1989) *Molecular Cloning: A Laboratory Manual* (2d ed., Cold Spring Harbor Laboratory Press, Plainview, New York). See also Innis *et al.*, eds. (1990) *PCR Protocols: A Guide to Methods and Applications* (Academic Press, New York); Innis and Gelfand, eds. (1995) *PCR Strategies* (Academic Press, New York); and Innis and Gelfand, eds. (1999) *PCR Methods Manual* (Academic Press, New York). Known methods of PCR include, but are not limited to, methods using paired primers, nested primers, single specific primers, degenerate primers, gene-specific primers, vector-specific primers, partially-mismatched primers, and the like.

In hybridization techniques, all or part of a known nucleotide sequence is used as a probe that selectively hybridizes to other corresponding nucleotide sequences present in a population of cloned genomic DNA fragments or cDNA fragments (i.e., genomic or cDNA libraries) from a chosen organism. The hybridization probes may be genomic DNA fragments, cDNA fragments, RNA fragments, or other oligonucleotides, and may be labeled with a detectable group such as ^{32}P, or any other detectable marker. Thus, for example, probes for hybridization can be made by labeling synthetic oligonucleotides based on, for example, the 50 kD gamma zein sequence of the invention. Methods for preparation of probes for hybridization and for construction of cDNA and genomic libraries are generally known in the art and are disclosed in Sambrook *et al.* (1989) *Molecular Cloning: A Laboratory Manual* (2d ed., Cold Spring Harbor Laboratory Press, Plainview, New York).

For example, the entire 50 kD gamma-zein, 18 kD alpha-globulin, or 50 kD legumin 1 sequence disclosed herein, or one or more portions thereof, may be used as a probe capable of specifically hybridizing to corresponding seed protein

sequences and messenger RNAs. To achieve specific hybridization under a variety of conditions, such probes include sequences that are unique among the seed protein sequences of the invention and are preferably at least about 40 nucleotides in length. Such probes may be used to amplify corresponding gamma-zein, alpha-globulin, and legumin 1 sequences from a chosen plant by PCR. This technique may be used to isolate additional coding sequences from a desired plant or as a diagnostic assay to determine the presence of coding sequences in a plant. Hybridization techniques include hybridization screening of plated DNA libraries (either plaques or colonies; see, for example, Sambrook *et al.* (1989) *Molecular Cloning: A Laboratory Manual* (2d ed., Cold Spring Harbor Laboratory Press, Plainview, New York).

Hybridization of such sequences may be carried out under stringent conditions. By "stringent conditions" or "stringent hybridization conditions" is intended conditions under which a probe will hybridize to its target sequence to a detectably greater degree than to other sequences (e.g., at least 2-fold over background). Stringent conditions are sequence-dependent and will be different in different circumstances. By controlling the stringency of the hybridization and/or washing conditions, target sequences that are 100% complementary to the probe can be identified (homologous probing). Alternatively, stringency conditions can be adjusted to allow some mismatching in sequences so that lower degrees of similarity are detected (heterologous probing). Generally, a probe is less than about 1000 nucleotides in length, preferably less than 500 nucleotides in length.

Typically, stringent conditions will be those in which the salt concentration is less than about 1.5 M Na ion, typically about 0.01 to 1.0 M Na ion concentration (or other salts) at pH 7.0 to 8.3 and the temperature is at least about 30°C for short probes (e.g., 10 to 50 nucleotides) and at least about 60°C for long probes (e.g., greater than 50 nucleotides). Stringent conditions may also be achieved with the addition of destabilizing agents such as formamide. Exemplary low stringency conditions include hybridization with a buffer solution of 30 to 35% formamide, 1 M NaCl, 1% SDS (sodium dodecyl sulphate) at 37°C, and a wash in 1X to 2X SSC (20X SSC = 3.0 M NaCl/0.3 M trisodium citrate) at 50 to 55°C. Exemplary moderate stringency conditions include hybridization in 40 to 45% formamide, 1.0 M NaCl, 1%

SDS at 37°C, and a wash in 0.5X to 1X SSC at 55 to 60°C. Exemplary high stringency conditions include hybridization in 50% formamide, 1 M NaCl, 1% SDS at 37°C, and a wash in 0.1X SSC at 60 to 65°C. Duration of hybridization is generally less than about 24 hours, usually about 4 to about 12 hours.

Specificity is typically the function of post-hybridization washes, the critical factors being the ionic strength and temperature of the final wash solution. For DNA-DNA hybrids, the T_m can be approximated from the equation of Meinkoth and Wahl (1984) *Anal. Biochem. 138*:267-284: $T_m = 81.5°C + 16.6 (\log M) + 0.41 (\%GC) - 0.61 (\%\ form) - 500/L$; where M is the molarity of monovalent cations, %GC is the percentage of guanosine and cytosine nucleotides in the DNA, % form is the percentage of formamide in the hybridization solution, and L is the length of the hybrid in base pairs. The T_m is the temperature (under defined ionic strength and pH) at which 50% of a complementary target sequence hybridizes to a perfectly matched probe. T_m is reduced by about 1°C for each 1% of mismatching; thus, T_m, hybridization, and/or wash conditions can be adjusted to hybridize to sequences of the desired identity. For example, if sequences with $\geq$90% identity are sought, the T_m can be decreased 10°C. Generally, stringent conditions are selected to be about 5°C lower than the thermal melting point (T_m) for the specific sequence and its complement at a defined ionic strength and pH. However, severely stringent conditions can utilize a hybridization and/or wash at 1, 2, 3, or 4°C lower than the thermal melting point (T_m); moderately stringent conditions can utilize a hybridization and/or wash at 6, 7, 8, 9, or 10°C lower than the thermal melting point (T_m); low stringency conditions can utilize a hybridization and/or wash at 11, 12, 13, 14, 15, or 20°C lower than the thermal melting point (T_m). Using the equation, hybridization and wash compositions, and desired T_m, those of ordinary skill will understand that variations in the stringency of hybridization and/or wash solutions are inherently described. If the desired degree of mismatching results in a T_m of less than 45°C (aqueous solution) or 32°C (formamide solution), it is preferred to increase the SSC concentration so that a higher temperature can be used. An extensive guide to the hybridization of nucleic acids is found in Tijssen (1993) *Laboratory Techniques in Biochemistry and Molecular Biology—Hybridization with Nucleic Acid Probes,* Part I,

Chapter 2 (Elsevier, New York); and Ausubel *et al.*, eds. (1995) *Current Protocols in Molecular Biology*, Chapter 2 (Greene Publishing and Wiley-Interscience, New York). See Sambrook *et al.* (1989) *Molecular Cloning: A Laboratory Manual* (2d ed., Cold Spring Harbor Laboratory Press, Plainview, New York).

Thus, isolated sequences that encode polypeptides that function as a seed protein and which hybridize under stringent conditions to the 50 kD gamma-zein, the 18 kD alpha-globulin protein, or the 50 kD legumin 1 sequence disclosed herein, or to fragments thereof, are encompassed by the present invention. Such sequences will be at least about 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% or more homologous with the disclosed sequence. That is, the sequence identity of sequences may range, sharing at least about 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% or more sequence identity.

The following terms are used to describe the sequence relationships between two or more nucleic acids or polynucleotides: (a) "reference sequence", (b) "comparison window", (c) "sequence identity", (d) "percentage of sequence identity", and (e) "substantial identity".

(a) As used herein, "reference sequence" is a defined sequence used as a basis for sequence comparison. A reference sequence may be a subset or the entirety of a specified sequence; for example, as a segment of a full-length cDNA or gene sequence, or the complete cDNA or gene sequence.

(b) As used herein, "comparison window" makes reference to a contiguous and specified segment of a polynucleotide sequence, wherein the polynucleotide sequence in the comparison window may comprise additions or deletions (i.e., gaps) compared to the reference sequence (which does not comprise additions or deletions) for optimal alignment of the two sequences. Generally, the comparison window is at least 20 contiguous nucleotides in length, and optionally can be 30, 40, 50, 100, or longer. Those of skill in the art understand that to avoid a high similarity to a reference sequence due to inclusion of gaps in the polynucleotide sequence a gap penalty is typically introduced and is subtracted from the number of matches.

Methods of alignment of sequences for comparison are well known in the art. Thus, the determination of percent sequence identity between any two sequences can be accomplished using a mathematical algorithm. Non-limiting examples of such mathematical algorithms are the algorithm of Myers and Miller (1988) *CABIOS 4*:11-17; the local homology algorithm of Smith *et al.* (1981) *Adv. Appl. Math. 2*:482; the homology alignment algorithm of Needleman and Wunsch (1970) *J. Mol. Biol. 48*:443-453; the search-for-similarity-method of Pearson and Lipman (1988) *Proc. Natl. Acad. Sci. 85*:2444-2448; the algorithm of Karlin and Altschul (1990) *Proc. Natl. Acad. Sci. USA 872*264, modified as in Karlin and Altschul (1993) *Proc. Natl. Acad. Sci. USA 90*:5873-5877.

Computer implementations of these mathematical algorithms can be utilized for comparison of sequences to determine sequence identity. Such implementations include, but are not limited to: CLUSTAL in the PC/Gene program (available from Intelligenetics, Mountain View, California); the ALIGN program (Version 2.0) and GAP, BESTFIT, BLAST, FASTA, and TFASTA in the Wisconsin Genetics Software Package, Version 8 (available from Genetics Computer Group (GCG), 575 Science Drive, Madison, Wisconsin, USA). Alignments using these programs can be performed using the default parameters. The CLUSTAL program is well described by Higgins *et al.* (1988) *Gene 73*:237-244 (1988); Higgins *et al.* (1989) *CABIOS 5*:151-153; Corpet *et al.* (1988) *Nucleic Acids Res. 16*:10881-90; Huang *et al.* (1992) *CABIOS 8*:155-65; and Pearson *et al.* (1994) *Meth. Mol. Biol. 24*:307-331. The ALIGN program is based on the algorithm of Myers and Miller (1988) *supra*. A PAM120 weight residue table, a gap length penalty of 12, and a gap penalty of 4 can be used with the ALIGN program when comparing amino acid sequences. The BLAST programs of Altschul *et al* (1990) *J. Mol. Biol. 215*:403 are based on the algorithm of Karlin and Altschul (1990) *supra*. BLAST nucleotide searches can be performed with the BLASTN program, score = 100, wordlength = 12, to obtain nucleotide sequences homologous to a nucleotide sequence encoding a protein of the invention. BLAST protein searches can be performed with the BLASTX program, score = 50, wordlength = 3, to obtain amino acid sequences homologous to a protein or polypeptide of the invention. To obtain gapped alignments for comparison

purposes, Gapped BLAST (in BLAST 2.0) can be utilized as described in Altschul *et al.* (1997) *Nucleic Acids Res. 25*:3389. Alternatively, PSI-BLAST (in BLAST 2.0) can be used to perform an iterated search that detects distant relationships between molecules. See Altschul *et al.* (1997) *supra*. When utilizing BLAST, Gapped BLAST, PSI-BLAST, the default parameters of the respective programs (e.g., BLASTN for nucleotide sequences, BLASTX for proteins) can be used. See http://www.ncbi.nlm.nih.gov. Alignment may also be performed manually by inspection.

Unless otherwise stated, sequence identity/similarity values provided herein refer to the value obtained using GAP version 10 using the following parameters: % identity using GAP Weight of 50 and Length Weight of 3; % similarity using Gap Weight of 12 and Length Weight of 4, or any equivalent program, aligned over the full length of the sequence. By "equivalent program" is intended any sequence comparison program that, for any two sequences in question, generates an alignment having identical nucleotide or amino acid residue matches and an identical percent sequence identity when compared to the corresponding alignment generated by GAP Version 10.

GAP uses the algorithm of Needleman and Wunsch (1970) *J. Mol. Biol.* 48: 443-453, to find the alignment of two complete sequences that maximizes the number of matches and minimizes the number of gaps. GAP considers all possible alignments and gap positions and creates the alignment with the largest number of matched bases and the fewest gaps. It allows for the provision of a gap creation penalty and a gap extension penalty in units of matched bases. GAP must make a profit of gap creation penalty number of matches for each gap it inserts. If a gap extension penalty greater than zero is chosen, GAP must, in addition, make a profit for each gap inserted of the length of the gap times the gap extension penalty. Default gap creation penalty values and gap extension penalty values in Version 10 of the Wisconsin Genetics Software Package for protein sequences are 8 and 2, respectively. For nucleotide sequences the default gap creation penalty is 50 while the default gap extension penalty is 3. The gap creation and gap extension penalties can be expressed as an integer selected from the group of integers consisting of from

0 to 200. Thus, for example, the gap creation and gap extension penalties can be 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65 or greater.

GAP presents one member of the family of best alignments. There may be many members of this family, but no other member has a better quality. GAP displays four figures of merit for alignments: Quality, Ratio, Identity, and Similarity. The Quality is the metric maximized in order to align the sequences. Ratio is the quality divided by the number of bases in the shorter segment. Percent Identity is the percent of the symbols that actually match. Percent Similarity is the percent of the symbols that are similar. Symbols that are across from gaps are ignored. A similarity is scored when the scoring matrix value for a pair of symbols is greater than or equal to 0.50, the similarity threshold. The scoring matrix used in Version 10 of the Wisconsin Genetics Software Package is BLOSUM62 (see Henikoff and Henikoff (1989) *Proc. Natl. Acad. Sci. USA* 89:10915).

(c) As used herein, "sequence identity" or "identity" in the context of two nucleic acid or polypeptide sequences makes reference to the residues in the two sequences that are the same when aligned for maximum correspondence over a specified comparison window. When percentage of sequence identity is used in reference to proteins it is recognized that residue positions which are not identical often differ by conservative amino acid substitutions, where amino acid residues are substituted for other amino acid residues with similar chemical properties (e.g., charge or hydrophobicity) and therefore do not change the functional properties of the molecule. When sequences differ in conservative substitutions, the percent sequence identity may be adjusted upwards to correct for the conservative nature of the substitution. Sequences that differ by such conservative substitutions are said to have "sequence similarity" or "similarity". Means for making this adjustment are well known to those of skill in the art. Typically this involves scoring a conservative substitution as a partial rather than a full mismatch, thereby increasing the percentage sequence identity. Thus, for example, where an identical amino acid is given a score of 1 and a non-conservative substitution is given a score of zero, a conservative substitution is given a score between zero and 1. The scoring of

conservative substitutions is calculated, e.g., as implemented in the program PC/GENE (Intelligenetics, Mountain View, California).

(d) As used herein, "percentage of sequence identity" means the value determined by comparing two optimally aligned sequences over a comparison window, wherein the portion of the polynucleotide sequence in the comparison window may comprise additions or deletions (i.e., gaps) as compared to the reference sequence (which does not comprise additions or deletions) for optimal alignment of the two sequences. The percentage is calculated by determining the number of positions at which the identical nucleic acid base or amino acid residue occurs in both sequences to yield the number of matched positions, dividing the number of matched positions by the total number of positions in the window of comparison, and multiplying the result by 100 to yield the percentage of sequence identity.

(e)(i) The term "substantial identity" of polynucleotide sequences means that a polynucleotide comprises a sequence that has at least 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% or more sequence identity compared to a reference sequence using one of the alignment programs described using standard parameters. One of skill in the art will recognize that these values can be appropriately adjusted to determine corresponding identity of proteins encoded by two nucleotide sequences by taking into account codon degeneracy, amino acid similarity, reading frame positioning, and the like. Substantial identity of amino acid sequences for these purposes normally means sequence identity of at least 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% or more.

Another indication that nucleotide sequences are substantially identical is if two molecules hybridize to each other under stringent conditions. Generally, stringent conditions are selected to be about 5°C lower than the thermal melting point (T_m) for the specific sequence at a defined ionic strength and pH. However, stringent conditions encompass temperatures in the range of about 1°C to about 20°C lower than the T_m, depending upon the desired degree of stringency as otherwise qualified herein. Nucleic acids that do not hybridize to each other under stringent conditions are still substantially identical if the polypeptides they encode are substantially

identical. This may occur, e.g., when a copy of a nucleic acid is created using the maximum codon degeneracy permitted by the genetic code. One indication that two nucleic acid sequences are substantially identical is when the polypeptide encoded by the first nucleic acid is immunologically cross reactive with the polypeptide encoded by the second nucleic acid.

(e)(ii) The term "substantial identity" in the context of a peptide indicates that a peptide comprises a sequence with at least 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% or more sequence identity to the reference sequence over a specified comparison window. Alignment can be conducted using the homology alignment algorithm of Needleman and Wunsch (1970) *J. Mol. Biol. 48*:443-453. An indication that two peptide sequences are substantially identical is that one peptide is immunologically reactive with antibodies raised against the second peptide. Peptides that are "substantially similar" comprise a sequence with at least 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% or more sequence identity or sequence similarity to the reference sequence over a specified comparison window. In this case residue positions that are not identical instead differ by conservative amino acid changes.

The 50 kD gamma-zein nucleotide sequence cloned from a maize endosperm cDNA library (Example 1) and disclosed in the present invention (SEQ ID NO:1) displays sequence similarity to the other two described corn gamma-zein genes, 27 kD gamma-zein (represented herein by GenBank Accession No. P04706) and 16 kD gamma-zein (represented herein by GenBank Accession No. AAA33523). The 50 kD gamma-zein was named due to its apparent molecular weight by migration in SDS-PAGE. This cDNA encodes a 295 amino acid protein and also shows sequence similarity to seed proteins of other plant species. For example, wheat alpha-gliadin (GenBank -ID:TAU51305, Accession No. U51305). The 50 kD gamma-zein DNA sequences isolated from different inbred lines showed an unusually low level of polymorphism. Only one single nucleotide polymorphism (SNP) (a 3 bp insertion) was detected along the entire cDNA sequence from DNA isolated from the inbred lines Mo17 and B73. The 50 kD gamma-zein gene has been located on chromosome 7, bin 7.03.

The 18 kD alpha-globulin nucleotide sequence was also cloned from a maize endosperm cDNA library (Example 5) and is disclosed in the present invention (SEQ ID NO:3). The 18 kD alpha-globulin was named due to its similarity to a rice seed globulin (rice alpha-globulin, GenBank Accession No. D50643). This cDNA encodes a 206 amino acid protein. Unlike the case of the 50 kD gamma-zein, there is no other maize gene known related to alpha-globulin in maize. The 18 kD alpha-globulin cDNA shows sequence similarity to seed proteins from other cereals including rice, wheat, and oats. Different maize inbred lines showed considerable allelism in the 18 kD alpha-globulin gene including SNP's. The 18 kD alpha-globulin gene has been located on chromosome 6, bin 6.05.

The 50 kD legumin 1 nucleotide sequence was also cloned from a maize endosperm cDNA library (Example 8) and is disclosed in the present invention (SEQ ID NO:5). The 50 kD legumin 1 was named due to its similarity to 11S globulins found in other plant species: the so-called legumins. The 50 kD legumin 1 appears to be encoded by a single gene in the maize genome. It belongs to the 11S globulin superfamily and is closely related to legumins from other cereals (also called glutenins in rice and wheat or globulins in oat) and dicot plants. The 50 kD legumin polypeptide sequence is missing the evolutionary conserved 11S globulin pro-protein proteolytic site (Asn-Gly bond between the acidic chain and basic chain legumin regions) which makes it unique among the legumin protein superfamily. This cDNA encodes a 483 amino acid protein with a predicted N-terminal endoplasmic reticulum import signal peptide of 36 amino acids. The 50 kD legumin 1 DNA sequences isolated from different inbred lines showed a considerable level of polymorphism. The 50 kD legumin 1 gene has been mapped to chromosome 6, Bin 6.01.

The chromosomal location of the genes corresponding to the three cDNA's of the present invention have been determined as stated above. Knowing the map position of a gene is important and useful if it correlates with a trait, as is the case for the encoded polypeptides of the present invention. Certain alleles of these genes can, for instance, have an impact on seed hardness, starch extractability, energy availability, etc as is described in detail *infra*. Considerable knowledge has been accumulated regarding the so called Quantitative Trait Loci (QTL). Linkage of a gene

to a QTL is of significance regarding the impact of this gene on the corresponding trait. Further, the map position can be used for marker assisted breeding, which is a very economical and time saving way to introduce alleles into elite germplasm. Alternatively, SNP's can also be used to screen a wide variety of germplasm for advantageous alleles.

The 50 kD gamma-zein protein of the present invention displays a high cysteine content and is therefore predicted to have a high number of disulfide bonds or high "disulfide status", as is observed for the other gamma-zein proteins. By "disulfide status" is intended the portion of cysteine residues within a protein that participate in disulfide bonds or disulfide bridges. Such disulfide bonds can be formed between the sulfur of a first cysteine residue and the sulfur of a second cysteine residue. It is recognized that such first and second cysteine residues can occur as part of a single polypeptide chain, or alternatively, can occur on separate polypeptide chains referred to herein as "inter-molecular disulfide bonds". When "disulfide status" is used in reference to a seed or part thereof, the "disulfide status" of such a seed or part thereof is the total disulfide status of the proteins therein.

The disulfide-rich, gamma-zein protein fraction in corn has been implicated as a major determinant of the poor amino acid content of this grain which contributes to its low nutrient content. In addition, as a result of the high-disulfide status of this gamma-zein fraction of corn endosperm it is also a significant contributor to the wet-milling properties of corn grain. For example, in the wet-milling process, the higher the number of disulfide bonds, the greater the requirement for chemical reductants to break these bonds and to maximize the release of starch granules. It is in this way that extensive disulfide bonding negatively impacts the process of wet-milling.

The intermolecular disulfide bridges of the gamma-zeins, along with the hydrophobic beta-zein, and alpha- and delta-zeins, are also important for the formation and maintenance of protein bodies. These protein bodies contribute to the physical properties of the grain that also affect the wet-milling process. In the wet-milling process, chemical reductants are required to break protein disulfide bonds to maximize starch yield and quality (Hoseney, 1994). The use in wet mills of odorous

chemical such as sulfur dioxide and bisulfite requires extensive precautions and poses significant environmental problems (May, 1987).

Similar to that described for a decrease in the number of disulfide bonds, a decrease in the number of protein bodies can also be expected to improve the efficiency of the wet-milling process. Zein proteins interact during formation of protein bodies (through intermolecular disulfide bonds and hydropobic interactions), and these interactions are important for the formation of proteolytically stable complexes. A decrease in the expression of two or three gamma-zein genes can be expected to have an additive effect on the reduction of protein bodies resulting in a corresponding improvement in wet-milling properties.

The wet-milling properties of the corn grain of the present invention can be analyzed using a small-scale simulated wet-milling process incorporating or leaving out a reducing agent (bisulfite) in the steep water as used by Eckhoff *et al.*, (1996, *Cereal Chem. 73*:54-57).

In addition to the positive impact that reducing agents have on the release of starch granules in the wet-milling process, it has also been shown that reducing agents can increase the dry matter digestibility of sorghum and corn and, thus, improve their feed properties. This result is supported by the results of data from *in vitro* digestibility assays described in the present invention (Examples 2-4) that demonstrate that reducing agents increase the dry matter digestibility or energy availability of corn. See also: Hamaker, B.R., *et al* 1987. Improving the in vitro protein digestibility of sorghum with reducing agents. *Proc. Natl. Acad. Sci.* USA 84: 626-628.

The "energy value", or "caloric value" of a feed or food, which is determined by energy density or gross energy (GE) content and by energy availability, is also termed "metabolizable energy (ME) content." (see Wiseman, J., and Cole, D.J.A. 1985.)

As used herein, "energy availability" means the degree to which energy-rendering nutrients are available to the animal, often referred to as energy conversion (ratio of metabolizable energy content to gross energy content). Energy availability can be determined with in vivo balance trials, in which excreta are collected by standard methodology (e.g., Sibbald, 1976; McNab and Blair, 1988; Morgan et al.,

1975). Energy availability is largely determined by nutrient digestibility in the gastro-intestinal tract, although other factors such as absorption and metabolic utilization also influence energy availability.

"Digestibility" is defined herein as the fraction of the feed or food that is not excreted as feces. It can be further defined as digestibility of specific components (such as energy or protein) by determining the concentration of these components in the foodstuff and in the excreta. Digestibility can be estimated using in vitro assays, which is routinely done to screen large numbers of different food ingredients and plant varieties. In vitro techniques, including assays with rumen inocula and/or enzymes for ruminant livestock (e.g., Tilley and Terry, 1963; Pell and Schofield) and various combinations of enzymes for monogastric animals reviewed in Boisen and Eggum (1991) are also useful techniques for screening transgenic materials for which only limited sample is available.

The enzyme digestible dry matter (EDDM) assay used in these experiments as an indicator of *in vivo* digestibility is known in the art and can be performed according to the methods described in Boisen and Fernandez (1997) *Animal Feed Science and Technology 68*:83-92, and Boisen and Fernandez (1995) *Animal Feed Science and Technology 51*:29-43; which are herein incorporated in their entirety by reference. These data indicate that reducing the number of disulfide bonds in the seed of sorghum and corn can increase the dry matter digestibility of grain from these crops. It is also likely that a decrease in the disulfide-status of other grains would have a similar positive effect on their digestibility properties.

Although seed with extensive disulfide bonding exhibits poor wet-milling properties and decreased dry matter digestibility, a high disulfide-status has also been correlated with increased seed hardness and improved dry-milling properties. In fact, the transcript level of the 50 kD maize gamma-zein gene has been shown to be largely affected in several opacity mutants (o2, o5, and o9) and in opaque hordothionin-12 (US Patent 5,990,389) corn. These data indicate that this 50 kD maize gamma zein is a good gene candidate for altering other grain quality traits such as grain hardness. Assays for seed hardness are well known in the art and include

such methods as those used in the present invention, described in Pomeranz *et al.* (1985) *Cereal Chemistry* 62:108-112; herein incorporated in its entirety by reference.

Based on its amino acid sequence, the 18 kD alpha-globulin can also be expected to have a high number of disulfide bonds and to participate in intermolecular protein cross-linking. For this reason, over-expression of the 18 kD alpha-globulin protein can be predicted to increase seed hardness. The ability to confer seed hardness is particularly useful in the case of soft kernel phenotypes that are induced by mutation or transgenic polypeptides. An increase in the levels of the 18 kD alpha-globulin can be used as a method for improving the dry-milling properties of soft kernel phenotypes.

In addition to its high cysteine content, the 18 kD alpha-globulin protein also possesses a relatively high percentage of the essential amino acids tryptophan (4.6% by weight, cysteine (5.1% by weight), and methionine (3.9% by weight). For this reason, transgenic over-expression of the 18 kD alpha-globulin protein can be expected to significantly increase the percentage of tryptophan and sulfur-containing amino acids in corn grain and, thus, increase the nutritional value of the grain.

The "nutritional value" of a feed or food is defined as the ability of that feed or food to provide nutrients to animals or humans. The nutritional value is determined by 3 factors: concentration of nutrients (protein & amino acids, energy, minerals, vitamins, etc.), their physiological availability during the processes of digestion, absorption and metabolism, and the absence (or presence) of anti-nutritional (e.g., toxic) compounds.

Similar to the 18 kD alpha-globulin, the 50 kD legumin 1 protein also possesses a relatively high percentage of essential amino acids. This protein contains 6.7%, 0.7%, 2.2%, 1.1%, 3.6%, and 2.7% by weight of lysine, tryptophan, methionine, cysteine, isoleucine, and threonine, respectively. For this reason, transgenic over-expression of the 50 kD legumin 1 protein can also be expected to increase the nutritional value of the grain.

In addition to its desirable amino acid content, the 50 kD legumin 1 protein is assembled differently than other legumin polypeptides. As a result of the missing proteolytic cleavage site, the 50 kD legumin 1 protein is not cleaved into acidic and

basic chains. Instead this legumin assembles into 9S polypeptide primers (presumably in the endoplasmic reticulum) and does not undergo assembly into 11S globulin hexamers. The assembly properties of this 50 kD legumin 1 polypeptide could contribute to unique food processing properties of protein extracts from seed expressing this protein. For example, the 50kD legumin 1 polypeptide could be ectopically expressed in soybean seed and protein isolates from corresponding soybean seed display altered functionalities such as solubility under acidic conditions, improved water-holding capacity and the like.

Another feature of the 50 kD legumin 1 polypeptide is a string of histidine residues that can function as a metal binding site. Native 50 kD legumin 1 polypeptide binds with high affinity to nickel chelation columns. This property can be used to purify corn legumin 1 in bulk from complex protein mixtures and to purify other polypeptides of interest through the production of fusion proteins. The metal chelation properties of the 50 kD legumin 1 polypeptide could also be of importance for bio-remediation or food health (antioxidant) applications.

It has also been demonstrated that proteolytic digestion of the alcohol-soluble seed protein fraction (prolamins) from wheat, barley, oats, and rye is known to give rise to biologically active, anti-nutritional peptides able to adversely affect the intestinal mucosa of coeliac patients (Silano and Vincenzi (1999) *Nahrung 43*:175-184). Furthermore, the alpha-, beta-, and gamma-gliadins present in the prolamin-like protein fraction of wheat are capable of inducing coeliac disease (Friis *et al.* (1994) *Clin. Chim. Acta. 231*:173-183). The alpha-gliadin and gamma-gliadin from wheat have also been identified as major allergens (Maruyama *et al.* (1998) *Eur. J. Biochem. 256*:604. For these reasons the methods of the present invention are also directed to the elimination or the reduction of the levels of at least one seed protein in wheat, barley, oats, or rye to produce a grain with eliminated or reduced anti-nutritional or allergenic properties.

The compositions of the invention are useful for modulating the levels of at least one seed protein in seeds. By "modulate" is defined herein as an increase or decrease in the level of a seed protein within seed of a genetically manipulated plant relative to the level of that protein in seed from the corresponding wild-type plant (i.e.,

a plant not genetically manipulated in accordance with the methods of the present invention). In a first embodiment, methods are particularly directed to reducing the level the 16 kD, the 27 kD protein and the 50 kD gamma-zein proteins to improve the nutritional value and industrial use of grain. A second embodiment is directed to the reduction or elimination of the alpha-, beta-, and gamma-gliadins of wheat, barley, rye, and oats to eliminate or ameliorate the anti-nutritional or allergenic effects of these proteins. In another embodiment, the levels of the alpha-globulin protein or the legumin 1 protein in plant seed are either increased or decreased to affect the nutritional value, or the hardness of the seed. Other embodiments of the invention include methods directed to screening for particular plant phenotypes based on antibodies specific for the polypeptides of the invention, or using SNP's of the nucleotide sequences of the invention.

Reduction of the level of the 16 kD, the 27 kD protein or the 50 kD gamma-zein proteins in plant seed can be used to improve the nutritional value and industrial use of such grain. By reducing the level of the 27 kD gamma-zein gene (Accession No. P04706) in maize seed, seed plants having improved amino acid composition can be obtained. Lysine content can be increased at least 10%, 15%, 20%, 30%, 40% or greater. The methods of the invention are also useful for producing grain that is more rapidly and extensively digested than grain with normal gamma-zein protein levels.

Because the 27 kD gamma-zein suppression trait is dominant or semi-dominant, improvements in grain digestibility can be obtained by introducing it into specific pollinators (i.e., high oil corn) using conventional methods and/or the top-cross technology found in US Patent No. 5,704,160. In addition, reducing the levels of other seed proteins, such as beta-zein, in conjunction with suppression of one or more gamma-zein genes can result in further grain improvement including lysine content and digestibility.

Thus, suppression of gamma-zein genes can be used to increase the nutritional value of seed, particularly by increasing the lysine content of the seed, and the digestibility of seed. Reduction in the gamma-zein levels in such seed can be at least about 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90% and up to 100%. Increases

in the lysine content of such seed can be at least about 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, and 50% or higher. Digestibility can be improved by at least 3%, 6%, 9%, 12%, 15%, 20% and greater.

Methods of the invention are also directed to the reduction or elimination of the expression of one or more specific prolamin-like proteins in the seed from wheat, barley, oats, and rye that are known to give rise to biologically active, anti-nutritional peptides. These proteins include, but are not limited to, the alpha-, beta-, and gamma-gliadins of wheat. Grain and grain products possessing reduced levels of these proteins would not possess such negative characteristics as the ability to induce coeliac disease and an allergic response.

It is noted that modifications made to the grain by the present invention do not compromise grain handling properties with respect to mechanical damage. Mechanical damage to grain is a well-described phenomenon (e.g., McKenzie, 1985) that contributes to dust in elevators and livestock facilities, and which may increase susceptibility to pests; Grain damage can be quantified and assessed by objective measures (e.g., Gregory *et al.*, 1991) such as kernel density and test weight. See also: McKenzie, B.A. 1985.

The invention also encompasses modulation of an 18 kD alpha-globulin protein or a legumin 1 protein to affect the nutritional value and/or the hardness of plant seed. A decrease in or an elimination of the expression of at least one of these proteins results in seed with decreased nutritional value. Such grain has applications for use in diet food products. Alternatively, an increase in the levels of these proteins in plant seed would result in an increase in the nutritional value of the seed. The levels of the maize 18 kD alpha-globulin protein (SEQ ID NO:4) can be increased in maize seed, resulting in seed that can be predicted to possess at least about 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 100%, 110%, 120%, 130%, 140%, 150%, and up to a 300% increase in tryptophan and sulfur-containing amino acids relative to grain of wild-type plants. The level of the 50 kD legumin 1 protein can be similarly increased in maize seed to increase the level of essential amino acids in the grain. Food products and feed based on such seed will have a higher nutritional value based on the increased levels of essential amino acids.

In addition to the increase in nutritional value, an increase in the level of the 18 kD alpha-globulin protein in plant seed can be predicted to result in grain possessing increased hardness. This is due to an increase in disulfide bond status relative to grain from wild-type plants, and has applications for improving the dry-milling properties of such modified grain. Introduction of this trait into corn plants with soft kernel phenotypes, particularly soft kernel phenotypes induced by the introduction of other transgenic polypeptides including, but not limited to, hordothionin 12 (US Patent 5,990,389), can ameliorate or eliminate the undesirable dry-milling properties of such soft kernel grain by increasing seed hardness.

In another embodiment, the levels of the 50 kD legumin 1 polypeptide are increased in plant seed for the purpose of increasing the metal chelating properties of the seed. The unique string of histidine residues present in the 50 kD legumin 1 polypeptide function as a metal chelating site. Products produced from such seed could be used for bio-remediation or in food health (antioxidant) applications.

Methods are provided for modulating the level of at least one seed protein in plant seed including, but not limited to, the 50 kD gamma-zein (SEQ ID NO:2), the 18 kD alpha-globulin (SEQ ID NO:4), the legumin 1 (SEQ ID NO:6), the 27 kD gamma-zein (Accession No. P04706), the 16 kD gamma-zein (Accession No. AAA33523), the15 kD beta-zein (Accession No. P06673), the gamma-kafarins, and the alpha-, beta-, and gamma-gliadins. The methods of the invention comprise the use of transgenic expression, antisense suppression, co-suppression, mutagenesis including transposon tagging, and biosynthetic competition, alone or in combination. Depending upon the intended goal, the level of at least one seed protein may be increased, decreased, or eliminated entirely as described below. Methods of the invention can be utilized to alter the level of any seed protein found within a particular plant species, including the alpha-, beta-, delta-, gamma-zeins of maize, and alpha-globulins of maize, the legumin 1 and other seed proteins of maize, rice and sorghum, and the alpha-, beta-, and gamma-gliadins of wheat, barley, rye, and oats. "Alter" and "modulate" are herein used interchangeably.

In many instances the seed coding sequences for use in the methods of the present invention, are provided in "expression cassettes" for expression in the plant of

interest. The expression cassette will include 5' and 3' regulatory sequences operably linked to at least one of the sequences of the invention. By "operably linked" is intended a functional linkage between a promoter and a second sequence, wherein the promoter sequence initiates and mediates transcription of the DNA sequence corresponding to the second sequence. Generally, operably linked means that the nucleic acid sequences being linked are contiguous and, where necessary to join two protein coding regions, contiguous and in the same reading frame. In the case where an expression cassette contains two protein coding regions joined in a contiguous manner in the same reading frame, the encoded polypeptide is herein defined as a "heterologous polypeptide" or a "chimeric polypeptide" or a "fusion polypeptide". The cassette may additionally contain at least one additional coding sequence to be co-transformed into the organism. Alternatively, the additional coding sequence(s) can be provided on multiple expression cassettes.

The methods of transgenic expression can be used to increase the level of at least one seed protein in grain. The methods of transgenic expression comprise transforming a plant cell with at least one expression cassette comprising a promoter that drives expression in the plant operably linked to at least one nucleotide sequence encoding a seed protein. Methods for expressing transgenic genes in plants are well known in the art.

The methods of transgenic co-suppression can be used to reduce or eliminate the level of at least one seed protein in grain. The methods of transgenic co-suppression comprise transforming a plant cell with at least one expression cassette comprising a promoter that drives expression in the plant operably linked to at least one nucleotide sequence transcript in the sense orientation encoding at least a portion of the seed protein of interest. By "co-suppression" is intended the use of nucleotide sequences in the sense orientation to suppress the expression of the corresponding endogenous genes in plants. Methods for suppressing gene expression in plants using nucleotide sequences in the sense orientation are known in the art. The methods generally involve transforming plants with a DNA construct comprising a promoter that drives expression in a plant operably linked to at least a portion of a nucleotide sequence that corresponds to the transcript of the endogenous

gene. Typically, such a nucleotide sequence has substantial sequence identity to the sequence of the transcript of the endogenous gene, at least about 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% or more sequence identity. See U.S. Patent Nos. 5,283,184 and 5,034,323; herein incorporated by reference.

The endogenous gene targeted for co-suppression may be a gene encoding any seed protein that accumulates as a seed protein in the plant species of interest, including, but not limited to, the seed genes noted above. For example, where the endogenous gene targeted for co-suppression is the 50 kD gamma-zein gene disclosed herein, co-suppression is achieved using an expression cassette comprising the 50 kD gamma-zein gene sequence, or variant or fragment thereof.

Additional methods of co-suppression are known in the art and can be similarly applied to the instant invention. These methods involve the silencing of a targeted gene by intron-spliced hairpin RNA's (see Smith *et al.* (2000) *Nature 407*:319-320, and Patent Applications WO 98/53083 and WO 99/53050).

The methods of antisense suppression can be used to reduce or eliminate the level of at least one seed protein in grain. The methods of antisense suppression comprise transforming a plant cell with at least one expression cassette comprising a promoter that drives expression in the plant cell operably linked to at least one nucleotide sequence that is antisense to a nucleotide sequence transcript of such a gamma-zein gene. By "antisense suppression" is intended as the use of nucleotide sequences that are antisense to nucleotide sequence transcripts of endogenous plant genes to suppress the expression of those genes in the plant.

Methods for suppressing gene expression in plants using nucleotide sequences in the antisense orientation are known in the art. The methods generally involve transforming plants with a DNA construct comprising a promoter that drives expression in a plant operably linked to at least a portion of a nucleotide sequence that is antisense to the transcript of the endogenous gene. Antisense nucleotides are constructed to hybridize with the corresponding mRNA. Modifications of the antisense sequences may be made as long as the sequences hybridize to and interfere with expression of the corresponding mRNA. In this manner, antisense

constructions having at least about 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% or more sequence identity to the corresponding antisense sequences may be used. Furthermore, portions, rather than the entire nucleotide sequence, of the antisense nucleotides may be used to disrupt the expression of the target gene. Generally, sequences of at least 50 nucleotides, 100 nucleotides, 200 nucleotides, or greater may be used.

The methods of transposon tagging can be used to reduce or eliminate the level of at least one seed protein in grain. The methods of transposon tagging comprise insertion of a transposon within an endogenous plant seed gene to reduce or eliminate expression of the seed protein. By "seed gene" is meant the gene that corresponds to the particular seed cDNA of interest. For example, by "50 kD gamma-zein gene" is meant the gene that corresponds to the cDNA set forth in SEQ ID NO:1.

In this method, a decrease or elimination of the expression of the seed protein of the invention is the goal, and insertion of a transposon within a regulatory region of this gene, in addition to, or rather than, an insertion within the seed-protein coding sequence, may result in decreased expression of the seed protein. For this reason, a transposon that is within an exon, intron, 5' or 3' untranslated sequence, a promoter, or any other regulatory sequence of, for example, the gamma-zein gene corresponding to the 50 kD gamma-zein cDNA of the invention, that results in decreased expression of the gamma-zein protein, is also an object of this embodiment.

Methods for the transposon tagging of specific genes in plants are well known in the art (see for example, Maes *et al.* (1999) *Trends Plant Sci. 4*:90-96; Dharmapuri and Sonti (1999) *FEMS Microbiol. Lett. 179*:53-59; Meissner *et al.* (2000) *Plant J. 22*:265-274; Phogat *et al.* (2000) *J. Biosci. 25*:57-63; Walbot (2000) *Curr. Opin. Plant Biol. 2*:103-107; Gai *et al.* (2000) *Nuc. Acids Res. 28*:94-96; Fitzmaurice *et al.* (1999) *Genetics 153*:1919-1928). In addition, the TUSC process for selecting Mu-insertions in selected genes has been described (Bensen *et al.* (1995) *Plant Cell* 7:75-84; Mena *et al.* (1996) *Science* 274:1537-1540; U.S. Patent No. 5,962,764, which is herein incorporated by reference).

Other methods for decreasing or eliminating the expression of endogenous genes are also known in the art and can be similarly applied to the instant invention. These methods include other forms of mutagenesis, such as ethyl methanesulfonate-induced mutagenesis, deletion mutagenesis, and fast neutron deletion mutagenesis used in a reverse genetics sense (with PCR) to identify plant lines in which the endogenous gene has been deleted (for examples of these methods see Ohshima, *et al.* (1998) *Virology 243*:472-481; Okubara *et al.* (1994) *Genetics 137*:867-874; Quesada *et al.* (2000) *Genetics 154*:421-436. In addition, a fast and automatable method for screening for chemically induced mutations, TILLING, (Targeting Induced Local Lesions In Genomes), using a denaturing HPLC or selective endonuclease digestion of selected PCR products is also applicable to the instant invention (see McCallum *et al.* (2000) *Nat. Biotechnol. 18*:455-457).

Methods of biosynthetic competition with other high-sulfur-containing proteins are used to reduce the levels of at least one seed protein in plant seed. The methods of biosynthetic competition comprise transforming plant cells with at least one expression cassette comprising a promoter that drives expression in the plant cell operably linked to at least one nucleotide sequence encoding a protein selected from the group consisting of delta-zeins, hordothionin 12, and other naturally occurring or engineered high-sulfur-containing proteins. In some cases the competing protein may possess a high lysine content in addition to a high sulfur content to further increase the nutritional value of the grain.

Biosynthetic competition of seed proteins with other sulfur–rich proteins occurs naturally. This natural process can be manipulated to reduce the levels of certain seed proteins, because the synthesis of some seed proteins is transcriptionally and/or translationally controlled by the nitrogen and/or sulfur supply in the developing seed. The expression of recombinant polypeptides, including the ectopic (transgenic) expression of seed proteins or other high-sulfur-, high-nitrogen-containing proteins, can have a substantial impact on intracellular nitrogen and sulfur pools. Thus, the expression of these proteins can result in suppression of the expression of other seed proteins such as, for example, the high-sulfur containing gamma-zein proteins.

Plant transformants containing a desired genetic modification as a result of any of the above described methods resulting in increased, decreased or eliminated expression of the seed protein of the invention can be selected by various methods known in the art. These methods include, but are not limited to, methods such as SDS-PAGE analysis, immunoblotting using antibodies which bind to the seed protein of interest, single nucleotide polymorphism (SNP) analysis, or assaying for the products of a reporter or marker gene, and the like.

Another embodiment is directed to the screening of transgenic maize plants for specific phenotypic traits conferred by the expression, or lack thereof, of the 50 kD gamma-zein, the 18 kD alpha-globulin, or the 50 kD legumin 1 polypeptides of the invention. The specific phenotypic traits for which this method finds use include, but are not limited to, all of those traits listed herein, *supra*. Maize lines can be screened for a particular phenotypic trait conferred by the presence or absence of the 50 kD gamma-zein, the 18 kD alpha-globulin, or the 50 kD legumin 1 protein using an antibody that binds selectively to one of these polypeptides. In this method, tissue from the maize line of interest is contacted with an antibody that selectively binds the seed-protein polypeptide for which the screen is designed. The amount of antibody binding is then quantified and is a measure of the amount of the seed-protein polypeptide present in the maize line. Methods of quantifying polypeptides by immunodetection in this manner are well known in the art.

An additional embodiment is directed to the use of the 50 kD legumin 1 protein to purify a polypeptide of interest based on the metal chelating properties of the 50 kD legumin 1 polypeptide. In this case recombinant DNA techniques known in the can be used to produce an expression cassette encoding a heterologous polypeptide consisting of the 50 kD legumin 1 polypeptide or a fragment thereof fused to a polypeptide of interest. The expression cassette can be introduced into either a eucaryotic or a bacterial host cell and the protein expressed in the host cell. The protein can then be isolated from the cells by an appropriate purification scheme using standard metal chelating column techniques such as high affinity nickel chelating columns that are commercially available. The legumin 1 nucleotide sequence can be fused to either

the N-terminus or C-terminus of the nucleotide sequence encoding the polypeptide of interest.

In the practice of certain specific embodiments of the present invention, a plant is genetically manipulated to have a suppressed or increased level of one or more seed proteins in seed and/or to ectopically express one or more seed or other high-sulfur, high-lysine-containing protein. Those of ordinary skill in the art realize that this can be accomplished in any one of a number of ways. For example, each of the respective coding sequences for such proteins can be operably linked to a promoter and then joined together in a single continuous fragment of DNA comprising a multigenic expression cassette. Such a multigenic expression cassette can be used to transform a plant to produce the desired outcome utilizing any of the methods of the invention including sense and antisense suppression and biosynthetic competition. Alternatively, separate plants can be transformed with expression cassettes containing one of the desired set of coding sequences. Transgenic plants resulting from any or a combination of the methods of the invention including transgenic expression, co-suppression, antisense suppression, mutagenesis including transposon tagging, and biosynthetic competition that express the desired activity can be selected by standard methods available in the art. These methods include, but are not limited to, methods such as immunoblotting using antibodies which bind to the proteins of interest, SNP analysis, or assaying for the products of a reporter or marker gene, and the like. Then, all of the desired coding sequences and/or transposon tagged sequences can be brought together into a single plant through one or more rounds of cross pollination utilizing the previously selected transformed plants as parents.

The seed coding sequences for use in the methods of the present invention are provided in expression cassettes for expression in the plant of interest. Such expression cassettes are provided with a plurality of restriction sites for insertion of the 50 kD gamma-zein, the 18 kD alpha-globulin, the 50 kD legumin 1 sequence or any other sequence of the present invention to be placed under the transcriptional regulation of the regulatory regions. The expression cassettes may additionally contain selectable marker genes.

The expression cassette will include in the 5'-3' direction of transcription, a transcriptional and translational initiation region, the 50 kD gamma-zein, the 18 kD alpha-globulin, or the 50 kD legumin 1 DNA sequence of the invention, and a transcriptional and translational termination region functional in plants. The transcriptional initiation region, the promoter, may be native or analogous or foreign or heterologous to the plant host. Additionally, the promoter may be the natural sequence or alternatively a synthetic sequence. By "foreign" is intended that the transcriptional initiation region is not found in the native plant into which the transcriptional initiation region is introduced. As used herein, a chimeric gene comprises a coding sequence operably linked to a transcription initiation region that is heterologous to the coding sequence.

While it may be preferable to express the sequences using heterologous promoters, the native promoter sequences may be used. Such constructs would change expression levels of the 50 kD gamma-zein, the 18 kD alpha-globulin, or the 50 kD legumin 1 gene in the plant or plant cell. Thus, the phenotype of the plant or plant cell is altered.

The termination region may be native with the transcriptional initiation region, may be native with the operably linked DNA sequence of interest, or may be derived from another source. Convenient termination regions are available from the Ti-plasmid of *A. tumefaciens*, such as the octopine synthase and nopaline synthase termination regions. See also Guerineau *et al.* (1991) *Mol. Gen. Genet. 262*:141-144; Proudfoot (1991) *Cell 64*:671-674; Sanfacon *et al.* (1991) *Genes Dev. 5*:141-149; Mogen *et al.* (1990) *Plant Cell 2*:1261-1272; Munroe *et al.* (1990) *Gene 91*:151-158; Ballas *et al.* (1989) *Nucleic Acids Res. 17*:7891-7903; and Joshi *et al.* (1987) *Nucleic Acid Res. 15*:9627-9639.

Where appropriate, for example, as in the case of engineered high-sulfur-containing proteins for the method of biosynthetic competition, the gene(s) may be optimized for increased expression in the transformed plant. That is, the genes can be synthesized using plant-preferred codons for improved expression. See, for example, Campbell and Gowri (1990) *Plant Physiol.* 92:1-11 for a discussion of host-preferred codon usage. Methods are available in the art for synthesizing plant-

preferred genes. See, for example, U.S. Patent Nos. 5,380,831, and 5,436,391, and Murray *et al.* (1989) *Nucleic Acids Res. 17*:477-498, herein incorporated by reference.

Additional sequence modifications are known to enhance gene expression in a cellular host. These include elimination of sequences encoding spurious polyadenylation signals, exon-intron splice site signals, transposon-like repeats, and other such well-characterized sequences that may be deleterious to gene expression. The G-C content of the sequence may be adjusted to levels average for a given cellular host, as calculated by reference to known genes expressed in the host cell. When possible, the sequence is modified to avoid predicted hairpin secondary mRNA structures.

The expression cassettes may additionally contain 5' leader sequences in the expression cassette construct. Such leader sequences can act to enhance translation. Translation leaders are known in the art and include: picornavirus leaders, for example, EMCV leader (Encephalomyocarditis 5' noncoding region) (Elroy-Stein *et al.* (1989) *Proc. Natl. Acad. Sci. USA 86*:6126-6130); potyvirus leaders, for example, TEV leader (Tobacco Etch Virus) (Gallie *et al.* (1995) *Gene 165(2)*:233-238), MDMV leader (Maize Dwarf Mosaic Virus) (*Virology 154*:9-20), and human immunoglobulin heavy-chain binding protein (BiP) (Macejak *et al.* (1991) *Nature 353*:90-94); untranslated leader from the coat protein mRNA of alfalfa mosaic virus (AMV RNA 4) (Jobling *et al.* (1987) *Nature 325*:622-625); tobacco mosaic virus leader (TMV) (Gallie *et al.* (1989) in *Molecular Biology of RNA*, ed. Cech (Liss, New York), pp. 237-256); and maize chlorotic mottle virus leader (MCMV) (Lommel *et al.* (1991) *Virology 81*:382-385). See also, Della-Cioppa *et al.* (1987) *Plant Physiol. 84*:965-968. Other methods known to enhance translation can also be utilized, for example, introns, and the like.

In preparing the expression cassette, the various DNA fragments may be manipulated, so as to provide for the DNA sequences in the proper orientation and, as appropriate, in the proper reading frame. Toward this end, adapters or linkers may be employed to join the DNA fragments or other manipulations may be involved to provide for convenient restriction sites, removal of superfluous DNA, removal of

restriction sites, or the like. For this purpose, *in vitro* mutagenesis, primer repair, restriction, annealing, resubstitutions, e.g., transitions and transversions, may be involved.

Generally, the expression cassette will comprise a selectable marker gene for the selection of transformed cells. Selectable marker genes are utilized for the selection of transformed cells or tissues. Marker genes include genes encoding antibiotic resistance, such as those encoding neomycin phosphotransferase II (NEO) and hygromycin phosphotransferase (HPT), as well as genes conferring resistance to herbicidal compounds, such as glufosinate ammonium, bromoxynil, imidazolinones, and 2,4-dichlorophenoxyacetate (2,4-D). See generally, Yarranton (1992) *Curr. Opin. Biotech. 3*:506-511; Christopherson *et al.* (1992) *Proc. Natl. Acad. Sci. USA 89*:6314-6318; Yao *et al.* (1992) *Cell 71*:63-72; Reznikoff (1992) *Mol. Microbiol. 6*:2419-2422; Barkley *et al.* (1980) in *The Operon*, pp. 177-220; Hu *et al.* (1987) *Cell 48*:555-566; Brown *et al.* (1987) *Cell 49*:603-612; Figge *et al.* (1988) *Cell 52*:713-722; Deuschle *et al.* (1989) *Proc. Natl. Acad. Aci. USA 86*:5400-5404; Fuerst *et al.* (1989) *Proc. Natl. Acad. Sci. USA 86*:2549-2553; Deuschle *et al.* (1990) *Science 248*:480-483; Gossen (1993) Ph.D. Thesis, University of Heidelberg; Reines *et al.* (1993) *Proc. Natl. Acad. Sci. USA 90*:1917-1921; Labow *et al.* (1990) *Mol. Cell. Biol. 10*:3343-3356; Zambretti *et al.* (1992) *Proc. Natl. Acad. Sci. USA 89*:3952-3956; Baim *et al.* (1991) *Proc. Natl. Acad. Sci. USA 88*:5072-5076; Wyborski *et al.* (1991) *Nucleic Acids Res. 19*:4647-4653; Hillenand-Wissman (1989) *Topics Mol. Struc. Biol. 10*:143-162; Degenkolb *et al.* (1991) *Antimicrob. Agents Chemother. 35*:1591-1595; Kleinschnidt *et al.* (1988) *Biochemistry 27*:1094-1104; Bonin (1993) Ph.D. Thesis, University of Heidelberg; Gossen *et al.* (1992) *Proc. Natl. Acad. Sci. USA 89*:5547-5551; Oliva *et al.* (1992) *Antimicrob. Agents Chemother. 36*:913-919; Hlavka *et al.* (1985) *Handbook of Experimental Pharmacology*, Vol. 78 (Springer-Verlag, Berlin); Gill *et al.* (1988) *Nature 334*:721-724. Such disclosures are herein incorporated by reference.

The above list of selectable marker genes is not meant to be limiting. Any selectable marker gene can be used in the present invention.

The use of the term "nucleotide constructs" herein is not intended to limit the present invention to nucleotide constructs comprising DNA. Those of ordinary skill in

the art will recognize that nucleotide constructs, particularly polynucleotides and oligonucleotides, comprised of ribonucleotides and combinations of ribonucleotides and deoxyribonucleotides may also be employed in the methods disclosed herein. Thus, the nucleotide constructs of the present invention encompass all nucleotide constructs that can be employed in the methods of the present invention for transforming plants including, but not limited to, those comprised of deoxyribonucleotides, ribonucleotides, and combinations thereof. Such deoxyribonucleotides and ribonucleotides include both naturally occurring molecules and synthetic analogues. The nucleotide constructs of the invention also encompass all forms of nucleotide constructs including, but not limited to, single-stranded forms, double-stranded forms, hairpins, stem-and-loop structures, and the like.

Furthermore, it is recognized that the methods of the invention may employ a nucleotide construct that is capable of directing, in a transformed plant, the expression of at least one protein, or at least one RNA, such as, for example, an antisense RNA that is complementary to at least a portion of an mRNA. Typically such a nucleotide construct is comprised of a coding sequence for a protein or an RNA operably linked to 5' and 3' transcriptional regulatory regions. Alternatively, it is also recognized that the methods of the invention may employ a nucleotide construct that is not capable of directing, in a transformed plant, the expression of a protein or an RNA.

In addition, it is recognized that methods of the present invention do not depend on the incorporation of the entire nucleotide construct into the genome, only that the plant or cell thereof is altered as a result of the introduction of the nucleotide construct into a cell. In one embodiment of the invention, the genome may be altered following the introduction of the nucleotide construct into a cell. For example, the nucleotide construct, or any part thereof, may incorporate into the genome of the plant. Alterations to the genome of the present invention include, but are not limited to, additions, deletions, and substitutions of nucleotides in the genome. While the methods of the present invention do not depend on additions, deletions, or substitutions of any particular number of nucleotides, it is recognized that such additions, deletions, or substitutions comprise at least one nucleotide.

The nucleotide constructs of the invention also encompass nucleotide constructs that may be employed in methods for altering or mutating a genomic nucleotide sequence in an organism, including, but not limited to, chimeric vectors, chimeric mutational vectors, chimeric repair vectors, mixed-duplex oligonucleotides, self-complementary chimeric oligonucleotides, and recombinogenic oligonucleobases. Such nucleotide constructs and methods of use, such as, for example, chimeraplasty, are known in the art. Chimeraplasty involves the use of such nucleotide constructs to introduce site-specific changes into the sequence of genomic DNA within an organism. See U.S. Patent Nos. 5,565,350; 5,731,181; 5,756,325; 5,760,012; 5,795,972; and 5,871,984; all of which are herein incorporated by reference. See also, WO 98/49350, WO 99/07865, WO 99/25821, and Beetham *et al.* (1999) *Proc. Natl. Acad. Sci. USA 96*:8774-8778; herein incorporated by reference.

A number of promoters can be used in the practice of the invention. The promoters can be selected based on the desired outcome. The nucleic acids can be combined with constitutive, tissue-preferred, or other promoters for expression in plants, more preferably a promoter functional during seed development.

Such constitutive promoters include, for example, the core promoter of the Rsyn7 promoter and other constitutive promoters disclosed in WO 99/43838; the core CaMV 35S promoter (Odell *et al.* (1985) *Nature 313*:810-812); rice actin (McElroy *et al.* (1990) *Plant Cell 2*:163-171); ubiquitin (Christensen *et al.* (1989) *Plant Mol. Biol. 12*:619-632 and Christensen *et al.* (1992) *Plant Mol. Biol. 18*:675-689); pEMU (Last *et al.* (1991) *Theor. Appl. Genet. 81*:581-588); MAS (Velten *et al.* (1984) *EMBO J. 3*:2723-2730); ALS promoter (U.S. Patent No. 5,659,026), and the like. Other constitutive promoters include, for example, U.S. Patent Nos. 5,608,149; 5,608,144; 5,604,121; 5,569,597; 5,466,785; 5,399,680; 5,268,463; and 5,608,142.

Chemical-regulated promoters can be used to modulate the expression of a gene in a plant through the application of an exogenous chemical regulator. Depending upon the objective, the promoter may be a chemical-inducible promoter, where application of the chemical induces gene expression, or a chemical-repressible promoter, where application of the chemical represses gene expression. Chemical-

inducible promoters are known in the art and include, but are not limited to, the maize In2-2 promoter, which is activated by benzenesulfonamide herbicide safeners, the maize GST promoter, which is activated by hydrophobic electrophilic compounds that are used as pre-emergent herbicides, and the tobacco PR-1a promoter, which is activated by salicylic acid. Other chemical-regulated promoters of interest include steroid-responsive promoters (see, for example, the glucocorticoid-inducible promoter in Schena *et al.* (1991) *Proc. Natl. Acad. Sci. USA 88*:10421-10425 and McNellis *et al.* (1998) *Plant J. 14(2)*:247-257) and tetracycline-inducible and tetracycline-repressible promoters (see, for example, Gatz *et al.* (1991) *Mol. Gen. Genet. 227*:229-237, and U.S. Patent Nos. 5,814,618 and 5,789,156), herein incorporated by reference.

Tissue-preferred promoters can be utilized to target enhanced protein expression within a particular plant tissue. Tissue-preferred promoters include Yamamoto *et al.* (1997) *Plant J. 12(2)*255-265; Kawamata *et al.* (1997) *Plant Cell Physiol. 38(7)*:792-803; Hansen *et al.* (1997) *Mol. Gen Genet. 254(3)*:337-343; Russell *et al.* (1997) *Transgenic Res. 6(2)*:157-168; Rinehart *et al.* (1996) *Plant Physiol. 112(3)*:1331-1341; Van Camp *et al.* (1996) *Plant Physiol. 112(2)*:525-535; Canevascini *et al.* (1996) *Plant Physiol. 112(2)*:513-524; Yamamoto *et al.* (1994) *Plant Cell Physiol. 35(5)*:773-778; Lam (1994) *Results Probl. Cell Differ. 20*:181-196; Orozco *et al.* (1993) *Plant Mol Biol. 23(6)*:1129-1138; Matsuoka *et al.* (1993) *Proc Natl. Acad. Sci. USA 90(20)*:9586-9590; and Guevara-Garcia *et al.* (1993) *Plant J. 4(3)*:495-505. Such promoters can be modified, if necessary, for weak expression.

"Seed-preferred" promoters include both "seed-specific" promoters (those promoters active during seed development such as promoters of seed storage proteins) as well as "seed-germinating" promoters (those promoters active during seed germination). See Thompson *et al.* (1989) *BioEssays 10*:108, herein incorporated by reference. Such seed-preferred promoters include, but are not limited to, Cim1 (cytokinin-induced message); cZ19B1 (maize 19 kD zein); milps (myo-inositol-1-phosphate synthase); and celA (cellulose synthase) (see the copending application entitled "Seed-Preferred Promoters," U.S. Application Serial No. 09/377,648, filed August 19, 1999, herein incorporated by reference). Gama-zein

is a preferred endosperm-specific promoter. Glb-1 is a preferred embryo-specific promoter. For dicots, seed-specific promoters include, but are not limited to, bean β-phaseolin, napin, β-conglycinin, soybean lectin, cruciferin, and the like. For monocots, seed-specific promoters include, but are not limited to, maize 15 kD zein, 22 kD zein, 27 kD zein, 10kD delta-zein, waxy, shrunken 1, shrunken 2, globulin 1, etc.

Where low level expression is desired, weak promoters will be used. Generally, by "weak promoter" is intended a promoter that drives expression of a coding sequence at a low level. By low level is intended at levels of about 1/1000 transcripts to about 1/100,000 transcripts to about 1/500,000 transcripts. Alternatively, it is recognized that weak promoters also encompasses promoters that are expressed in only a few cells and not in others to give a total low level of expression. Where a promoter is expressed at unacceptably high levels, portions of the promoter sequence can be deleted or modified to decrease expression levels.

Such weak constitutive promoters include, for example, the core promoter of the Rsyn7 promoter (WO 99/43838), the core 35S CaMV promoter, and the like. Other constitutive promoters include, for example, U.S. Patent Nos. 5,608,149; 5,608,144; 5,604,121; 5,569,597; 5,466,785; 5,399,680; 5,268,463; and 5,608,142. See also, the copending application entitled "Constitutive Maize Promoters," U.S. Application Serial No. 09/257,584, filed February 25, 1999, and herein incorporated by reference.

Methods of the invention can be utilized to alter the level of at lease one seed in seed from any plant species of interest. Plants of particular interest include grain plants that provide seeds of interest including grain seeds such as corn, wheat, barley, rice, sorghum, rye, oats, etc. The present invention may be used for many plant species, including, but not limited to, monocots and dicots. Examples of plant species of interest include, but are not limited to, corn (*Zea mays*), *Brassica* sp. (e.g., *B. napus, B. rapa, B. juncea*), particularly those *Brassica* species useful as sources of seed oil, alfalfa (*Medicago sativa*), rice (*Oryza sativa*), rye (*Secale cereale*), sorghum (*Sorghum bicolor, Sorghum vulgare*), millet (e.g., pearl millet (*Pennisetum glaucum*), proso millet (*Panicum miliaceum*), foxtail millet (*Setaria italica*), finger millet (*Eleusine*

coracana)), sunflower (*Helianthus annuus*), safflower (*Carthamus tinctorius*), wheat (*Triticum aestivum*), soybean (*Glycine max*), oats, and barley.

Transformation protocols as well as protocols for introducing nucleotide sequences into plants may vary depending on the type of plant or plant cell, i.e., monocot or dicot, targeted for transformation. Suitable methods of introducing nucleotide sequences into plant cells and subsequent insertion into the plant genome include microinjection (Crossway *et al.* (1986) *Biotechniques 4*:320-334), electroporation (Riggs *et al.* (1986) *Proc. Natl. Acad. Sci. USA 83*:5602-5606, *Agrobacterium*-mediated transformation (Townsend *et al.*, U.S. Patent No. 5,563,055; Zhao *et al.*, U.S. Patent No. 5,981,840; Cai et al., US Patent Application No. 09/056,418), direct gene transfer (Paszkowski *et al.* (1984) *EMBO J. 3*:2717-2722), and ballistic particle acceleration (see, for example, Sanford *et al.*, U.S. Patent No. 4,945,050; Tomes *et al.*, U.S. Patent No. 5,879,918; Tomes *et al.*, U.S. Patent No. 5,886,244; Bidney *et al.*, U.S. Patent No. 5,932,782; Tomes *et al.* (1995) "Direct DNA Transfer into Intact Plant Cells via Microprojectile Bombardment," in *Plant Cell, Tissue, and Organ Culture: Fundamental Methods*, ed. Gamborg and Phillips (Springer-Verlag, Berlin); and McCabe *et al.* (1988) *Biotechnology 6*:923-926). Also see Weissinger *et al.* (1988) *Ann. Rev. Genet. 22*:421-477; Sanford *et al.* (1987) *Particulate Science and Technology 5*:27-37 (onion); Christou *et al.* (1988) *Plant Physiol. 87*:671-674 (soybean); McCabe *et al.* (1988) *Bio/Technology 6*:923-926 (soybean); Finer and McMullen (1991) *In Vitro Cell Dev. Biol.* 27P:175-182 (soybean); Singh *et al.* (1998) *Theor. Appl. Genet. 96*:319-324 (soybean); Datta *et al.* (1990) *Biotechnology 8*:736-740 (rice); Klein *et al.* (1988) *Proc. Natl. Acad. Sci. USA 85*:4305-4309 (maize); Klein *et al.* (1988) *Biotechnology 6*:559-563 (maize); Tomes, U.S. Patent No. 5,240,855; Buising *et al.*, U.S. Patent Nos. 5,322,783 and 5,324,646; Tomes *et al. (1995)* "Direct DNA Transfer into Intact Plant Cells via Microprojectile Bombardment," in *Plant Cell, Tissue, and Organ Culture: Fundamental Methods*, ed. Gamborg (Springer-Verlag, Berlin) (maize); Klein *et al.* (1988) *Plant Physiol. 91*:440-444 (maize); Fromm *et al.* (1990) *Biotechnology 8*:833-839 (maize); Hooykaas-Van Slogteren *et al.* (1984) *Nature (London) 311*:763-764; Bowen *et al.*, U.S. Patent No. 5,736,369 (cereals); Bytebier *et al.* (1987) *Proc. Natl. Acad. Sci. USA*

84:5345-5349 (Liliaceae); De Wet *et al.* (1985) in *The Experimental Manipulation of Ovule Tissues,* ed. Chapman *et al.* (Longman, New York), pp. 197-209 (pollen); Kaeppler *et al.* (1990) *Plant Cell Reports 9*:415-418 and Kaeppler *et al.* (1992) *Theor. Appl. Genet. 84*:560-566 (whisker-mediated transformation); D'Halluin *et al.* (1992) *Plant Cell 4*:1495-1505 (electroporation); Li *et al.* (1993) *Plant Cell Reports 12*:250-255 and Christou and Ford (1995) *Annals of Botany 75*:407-413 (rice); Osjoda *et al.* (1996) *Nature Biotechnology 14*:745-750 (maize via *Agrobacterium tumefaciens*); all of which are herein incorporated by reference.

The methods of the invention involve introducing a nucleotide construct into a plant. By "introducing" is intended presenting to the plant the nucleotide construct in such a manner that the construct gains access to the interior of a cell of the plant. The methods of the invention do not depend on a particular method for introducing a nucleotide construct to a plant, only that the nucleotide construct gains access to the interior of at least one cell of the plant. Methods for introducing nucleotide constructs into plants are known in the art including, but not limited to, stable transformation methods, transient transformation methods, and virus-mediated methods.

By "stable transformation" is intended that the nucleotide construct introduced into a plant integrates into the genome of the plant and is capable of being inherited by progeny thereof. By "transient transformation" is intended that a nucleotide construct introduced into a plant does not integrate into the genome of the plant.

The nucleotide constructs of the invention may be introduced into plants by contacting plants with a virus or viral nucleic acids. Generally, such methods involve incorporating a nucleotide construct of the invention within a viral DNA or RNA molecule. It is recognized that the protein of interest of the invention may be initially synthesized as part of a viral polyprotein, which later may be processed by proteolysis *in vivo* or *in vitro* to produce the desired recombinant protein. Further, it is recognized that promoters of the invention also encompass promoters utilized for transcription by viral RNA polymerases. Methods for introducing nucleotide constructs into plants and expressing a protein encoded therein, involving viral DNA or RNA molecules, are known in the art. See, for example, U.S. Patent Nos.

5,889,191, 5,889,190, 5,866,785, 5,589,367 and 5,316,931; herein incorporated by reference.

The cells that have been transformed may be grown into plants in accordance with conventional ways, under plant forming conditions. See, for example, McCormick *et al.* (1986) *Plant Cell Reports* 5:81-84. These plants may then be grown, and either pollinated with the same transformed strain or different strains, and the resulting hybrid having expression of the desired phenotypic characteristic identified. Two or more generations may be grown to ensure that expression of the desired phenotypic characteristic is stably maintained and inherited and then seeds harvested to ensure expression of the desired phenotypic characteristic has been achieved.

In addition, the desired genetically altered trait can be bred into other plant lines possessing other desirable characteristics using conventional breeding methods and/or top-cross technology. The top-cross method is taught in US Pat. No. 5,704,160 herein incorporated in its entirety by reference.

Methods for cross pollinating plants are well known to those skilled in the art, and are generally accomplished by allowing the pollen of one plant, the pollen donor, to pollinate a flower of a second plant, the pollen recipient, and then allowing the fertilized eggs in the pollinated flower to mature into seeds. Progeny containing the entire complement of heterologous coding sequences of the two parental plants can be selected from all of the progeny by standard methods available in the art as described *infra* for selecting transformed plants. If necessary, the selected progeny can be used as either the pollen donor or pollen recipient in a subsequent cross pollination.

Existing maize strains possessing desirable traits can be engineered to provide increased energy availability with the methods of this invention. While the methods of the invention do not depend on any particular biological mechanism experimental results indicate that the increased energy availability of the corn kernel genetically modified to contain reduced levels of gamma-zein proteins is likely a result of reduced grain disulfide content. It has been shown that the response in digestibility

to the treatment of grain with DTT is inversely related to the digestibility of untreated grain (Boisen and Eggum, 1991).

Digestibility of immature grain (grain at late dough or silage maturity stage) is equally improved by pretreatment with reducing agents (DTT) as mature grain. The same can be expected for low gamma zein corn as the effects of DTT pretreatment, and low gamma zein corn, on digestibility are virtually the same. Improvements in digestibility of immature grain through the methods of the present invention can be extrapolated to improvements in digestibility of silage - about half of which consists of immature grain. The improvements in digestibility with DTT pretreatment is inversely related to the intrinsic digestibility of untreated grain. For this reason, corn lines of low intrinsic digestibility (i.e. high gamma zein levels) can be expected to be more amenable to genetic modification through the method of the invention than those of higher digestibility (i.e. low gamma zein levels). This aspect of the invention enables those of skill in the art of breeding to make rapid advances in introgressing a low gamma zein trait into the appropriate elite germplasm.

This invention allows for the improvement of grain properties such as increased digestibility/nutrient availability, nutritional value, silage quality, and efficiency of wet or dry milling in maize strains already possessing other desirable characteristics.

Corn grain with reduced gamma-zein protein content offers the following advantages:

First, ground corn grain with a reduced gamma-zein protein content offer increased energy availability and protein digestibility to monogastric livestock (see Example 2). "Monogastric animals" include but are not limited to: pigs, poultry, horses, dogs, cats, rabbits and rodents.

It can be deduced from analysis of the *in vitro* experimental data provided herein that the corn grain from maize genetically altered to contain reduced gamma-zein protein levels will have a 5% increase in metabolizable energy for poultry and pigs. Using this assumption the following replacement value can be assigned to the high energy availability trait in grain resulting from the gamma-zein gene knock-out. Five percent of 1665 kcal/lb equals about 83 kcal/lb. Taking into account that a

bushel of corn contains 56-60 lbs, the 83 kcal/lb difference amounts to a gain of 4650-5000 kcal/bu. This difference in available energy is equivalent to 1.3-1.4 lb fat, which, at 12cts/lb, is worth 15-17 cents per bushel.

Second, corn grain with a reduced gamma-zein protein content possess improved ruminant (e.g.: cattle sheep, and goats) feed quality through increased digestibility (see Example 2). Grain is fed to ruminants in minimally processed form, and the rigid protein structure of corn endosperm has been shown to constitute a large impediment to microbial digestion in the rumen, which can be partly overcome by predigestion with protease (McAllister *et al.* (1993) *J. Anim. Sci. 71*:205-212). A reduced gamma-zein protein content imparts a similar or even larger improvement to ruminal digestion of whole corn.

Third, corn grain with reduced levels of gamma-zein proteins has an increased response to feed processing. The nutrient availability from whole corn grain can be increased by extensive processing (steam-flaking or extrusion) resulting in starch gelatinization and protein disulfide bond reduction (Blackwood and Richardson, 1994). The response to processing is sometimes lower than expected. The heat and/or shearing force applied during processing causes rearrangements of protein disulfide bonds, which may partly counteract the improvement in digestibility resulting from starch gelatinization. The response to steam-flaking of corn and sorghum grain is negatively correlated with protein disulfide content (Blackwood and Richardson, 1994). For low gamma zein corn or low gamma kafarin sorghum the extent of disulfide rearrangements during processing is reduced, which allows for a more uniform response to steam-flaking, and which can be expected to reduce the energy required in steam-flaking or grinding processes.

Fourth, corn grain with a lower gamma-zein protein content has improved silage quality for dairy cattle, especially for silage harvested at late maturity. Although silage is harvested at earlier maturity than grain, a certain degree of dry-down (and protein disulfide formation) has already occurred by the time the crop is ensiled, especially under dry and hot conditions. Our work has shown that pretreatment with a reducing agent of immature, dough-stage corn kernels, sampled at silage maturity, resulted in drastically improved *in vitro* digestibility, a strong indication that the protein

disulfide imposed barriers to digestion had already been established. (data in Example 2) Hence, the digestibility of the "yellow portion" of corn silage can be expected to be higher for grain with a reduced gamma-zein protein content. Increased digestibility will be especially notable in the case of silage made from mature corn and for high-yielding dairy cows in which high passage rates do not allow for extensive ruminal digestion.

Fifth, corn grain with a reduced gamma-zein protein content will have an increased efficiency of wet milling. An increase in wet-milling efficiency and starch recovery can be expected due to the lower disulfide content of grain with reduced gamma-zein protein content. Efficiencies in the processes of wet milling also include reduced steeping time and/or reduced need for chemical reductants such as sulfur dioxide and sodium bisulfite. The use of fewer chemicals will improve wet-milling economics and reduce environmental pollution.

EXAMPLES

Maize lines (transgenic and transposon-mutagenized) have been developed with increased or decreased levels of specific endosperm proteins. For several of the obtained lines, experimental evidence indicates that the introduced changes result in improved grain properties.

Example 1: Cloning and Transgenic Co-suppression of a Novel Maize 50 kD Gamma-Zein.

A 50 kD gamma-zein nucleotide sequence was cloned from a maize endosperm cDNA library (mid and late development). Based on EST numbers 50 kD gamma-zein transcripts are relatively abundant (compared to other seed protein transcripts) and represent approximately 0.5% of the endosperm mRNA during mid development. A large variation in the abundance of 50 kD gamma-zein transcripts has been observed between different inbred lines (transcript profiling results). The 50 kD gamma-zein gene has been located on chromosome 7, bin 7.03

The 50kD gamma-zein cDNA sequences isolated from different inbred lines show an unusually low level of polymorphism. Only one SNP (a 3bp insertion) was detected along the entire cDNA sequence from DNA isolated from the inbred lines Mo17 and B73 (the SNP is bold and in lower case). See also SEQ ID NOS: 7 and 8.

50 kD gamma-zein, B73
partialCCAGCAGCAGCAACACCAACAACAACAAGTTCACATGCAACCACAAAAAC
ATCAGCAACAACAAGAAGTTCATGTTCAACAACAACAACAACAACCGCAGCACC
AACAACAACAACAACAACA**aca**GCACCAACAACAACATCAATGTGAAGGCCAACA
ACAACATCACCAACAATCACAAGGCCATGTGCAACAACACGAACAGAGCCATGA
GCAACACCAAGGACAGAGCCATGAGCAACAACATCAACAACAATTCCAGGGTCA
TGACAAGCAGCAACAACCACAACAGCCTCAGCAATATCAGCAGGGCCAGGAAAA
ATC

50 kD gamma-zein, Mo17 partial
CCAGCAGCAGCAACACCAACAACAACAAGTTCACATGCAACCACAAAAACATCA
GCAACAACAAGAAGTTCATGTTCAACAACAACAACAACAACCGCAGCACCAACA
ACAACAACAACAACA***GCACCAACAACAACATCAATGTGAAGGCCAACAACAA
CATCACCAACAATCACAAGGCCATGTGCAACAACACGAACAGAGCCATGAGCAA
CACCAAGGACAGAGCCATGAGCAACAACATCAACAACAATTCCAGGGTCATGAC
AAGCAGCAACAACCACAACAGCCTCAGCAATATCAGCAGGGCCAGGAAAAATC

The 50-kD gamma zein transformation event described herein was one of various high-digestibility events produced. The event was generated with a construct containing the 27kD gamma zein promoter, 50 kD gamma zein ORF in sense orientation, and 27-kD gamma zein terminator using particle bombardment. It was found to be reduced in all known gamma zein proteins, i.e., 50 kD-, 27 kD-, and 16 kD-gamma zein. Protein gel & 50 kD gamma zein Western blots of segregating CS50 events were performed to confirm co-suppression. The kernel phenotype of the transgenic seed was normal (i.e., vitreous).

Segregating kernels from transgenic corn co-suppressed in 50 kD gamma zein were ground to a fine meal and subjected to the monogastric in vitro digestibility assay to determine Enzyme Digestible Dry Matter (EDDM). EDDM of 50 kD gamma zein co-suppressed grain was improved by 3.0 percentage units. An overnight soak in 10 mM of the strong reducing agent dithiothreietol (DTT), known to maximize in

vitro digestibility, improved digestibility slightly beyond that reached with 50kD gamma zein co-suppression (by 1.4 percentage units.

Example 2: Transgenic Co-suppression of 27 kD Gamma-Zein.

Events in which the expression level of 27 kD gamma-zein protein was reduced to less than 5% of wild-type as determined by SDS-PAGE and immunoblotting were obtained with two transgenes. The endosperm protein profiles of grain in which the 27 kD gamma-zein gene was co-suppressed showed an additional reduction of approximately 60% in the level of the 16 kD gamma-zein protein and an approximate five-fold increase in the level of the hydrophobic l5 kD beta-zein protein. The overall lysine content in this grain increased by 15-30. Even with the significant decrease in high disulfide containing gamma-zein proteins, grain from these events showed a normal (vitreous) phenotype and were of unaltered test weight and hardness. This result was unexpected as the decrease in the disulfide content, and specifically the decrease in gamma zein, might have been expected to result in grain with a soft phenotype (see Lopez and Larkins, 1991). Assays for seed hardness are well known in the art and include such methods as those used in the present invention, described in Pomeranz *et al.* (1985) *Cereal Chemistry 62*:108-112, herein incorporated in its entirety by reference.

The co-suppression trait is shown to be dominant. Various normal and transgenic maize lines, as well as commercial hybrids, have been pollinated with pollen from the gamma-zein co-suppressing events with the result of total suppression of gamma-zein protein in the hemizygous endosperm as determined by SDS-PAGE and immunoblotting. Therefore, the gamma-zein gene co-suppression trait can be introduced into specific pollinators (i.e., high oil corn) using conventional methods and/or the top-cross technology found in US Patent No. 5,704,160.

T_3-segregating grain was phenotyped for gamma-zein protein levels and were divided into two samples, one with wild-type gamma-zein protein levels and a second with reduced gamma-zein protein levels (less than 10% of wild-type). Ground corn from both samples was subjected to an *in vitro* energy availability assay. The enzyme digestible dry matter (EDDM) assay used in these experiments as an

indicator of *in vivo* digestibility, is known in the art and was performed using enzymes, buffers, and digestion conditions described in Boisen and Fernandez (1997) *Animal Feed Science and Technology 68*:83-92; and Boisen and Fernandez (1995) *Animal Feed Science and Technology 51*:29-43, which are herein incorporated in their entirety by reference. The results clearly indicated that ground corn from gamma-zein co-suppressed grain were more rapidly and extensively digested than corn with normal gamma-zein protein levels, by as much as 20% at the 4 hour time point.

The role of disulfide bridges in the digestion of corn was investigated in gamma-zein gene co-suppressed versus control grain. As expected, pretreatment with a strong reducing agent (10 mM DTT) increased the enzyme digestible dry matter (EDDM) level (4 hour digestion) of control grain by 16% but not that of the gamma-zein gene co-suppressed grain. A similar result was observed for various low gamma-zein TopCross hybrids (e.g.: with grain hybrids 3394 and 32J55). Hence, the impact of DTT on digestibility apparently involves the reduction of disulfides of cysteine residues in gamma-zein proteins. Phenotyped kernel samples (those with normal levels of 27 kD gamma-zein protein and those with low levels of 27 kD gamma-zein protein) from segregating ears from the same events were analyzed using a small-scale simulated wet-milling process incorporating or leaving out a reducing agent (bisulfite) in the steep water (Eckhoff *et al.*, (1996) *Cereal Chem.* 73:54-57). Similar to the digestibility assay, the reductant had a lesser impact on starch extractability in grain containing low levels of 27 kD gamma-zein protein compared to wild-type grain.

Rumen *in situ* dry matter digestibility of co-suppressed (i.e. low) 27 kD gamma zein corn in a top-cross onto Pioneer grain hybrid 3394 was compared with a control top-cross and with the grain parent. Coarsely ground mature grain samples were weighed into pre-tared nylon bags (4 replicates each). The bags were sealed and placed in the rumen of a fistulated steer for 18hrs, then washed to remove microbial mass, ovendried, and weighed. Ruminal digestibility of the low gamma zein grain was 18% higher than the control top-cross and 10% higher than that of the grain parent. See also: Nocek, J.E. 1988. In situ and other methods to estimate ruminal protein and energy digestibility. *J. Dairy Sci.* 71:2051-2069.

The same samples subjected to the in situ digestion procedure were also evaluated by an automated *in vitro* gas production method as described by Pell and Schofield (1993).

The *in vitro* gas production curve comparing low gamma zein topcross to the grain parent and a control cross showed a higher gas production volume for the low gamma zein topcross, corresponding to a more rapid and extensive digestion of the low gamma-zein grain in rumen fluid. Coarsely ground mature grain samples were weighed into fermentation flasks (9 replicates each). The flasks were inoculated with buffered rumen fluid and incubated at 38°C for 24 hrs, during which the volume of the fermentation gas was automatically recorded. Average gas production was clearly higher for low gamma zein topcross than for each of the two controls.

Immature kernels of various wild-type inbreds & hybrids, sampled at various stages of seed development and maturation, consistently respond to DTT pretreatment in the monogastric in vitro assay when sampled 1 month after pollination or later. The improvement in digestibility with DTT points at a consistent inhibitory role of protein disulfide bonds on digestibility of wild-type kernels from about 28 DAP onwards. From these results one can conclude that kernels harvested at dough stage or silage maturity (approximately 40-45 DAP) would benefit from reduced gamma zein levels. We also applied DTT pretreatment prior to monogastric in vitro digestion of 27 kD gamma zein co-suppressed immature kernels (33 DAP), with no apparent effect, similar to our observations for low gamma zein mature grain. Given the response to DTT for wild-type immature kernels from 28 DAP through maturity, the lack of DTT response for low gamma zein kernels of any maturity, and the observed improvements in ruminal digestibility of mature low gamma zein grain, one can deduce, with very high likelihood, that ruminal digestibility of silage maturity kernels will be improved with gamma zein reduction.

Co-suppressed (i.e. low) 27 kD gamma zein corn produced as a top-cross onto Pioneer grain hybrid 3394 was compared with a control top-cross and with the grain parent in a chicken feeding trial. A 21-d chick growth trial was performed with digestibility measurements, which demonstrated increased (by 2 percentage units) in vivo dry matter digestibility and increased energy conversion efficiency (by 2%) for

the low gamma zein topcross. In addition, in vivo protein digestibility was improved by 9 percentage units (from 69 to 78%), representing a 13% increase. The increase in protein digestibility resulted in a 29% decrease in nitrogen excretion into the environment.

The same low gamma zein topcross was also compared with the control topcross in a pig in vivo digestion trial. Metabolizable Energy content of the low gamma zein topcross amounted to 3646 kcal/kg, 73 kcal (or 2%) higher than the control topcross. Protein digestibility was improved from 75.8 to 79.8% for the low gamma zein topcross. This represents a 5% improvement in protein digestibility, and a 15% reduction in nitrogen excretion into the environment.

Example 3: Suppression of 27 kD Gamma-Zein through Interruption of the 27 kD Gamma-Zein Gene by Transposon Tagging.

A maize line containing a Mu-insertion in the 27 kD gamma-zein gene was selected from the Pioneer TUSC collection using the TUSC process (Bensen *et al.* (1995) *Plant Cell* 7:75-84; Mena *et al.* (1996) *Science* 274:1537-1540; U.S. Patent No. 5,962,764) with the 27 kD gamma-zein gene specific primers. Homozygous seed with the Mu-allele showed an absolute suppression of 27 kD gamma-zein protein. The trait has been stable in backcrosses to inbred lines. Again, seed are normal (vitreous) and have an unaltered test weight. The lysine content of the grain was increased 15-30% compared to wild-type grain. A maize line containing a Mu-insertion in the 27 kD gamma-zein gene was used.

Grain from progeny of this TUSC line has been tested in the *in vitro* digestibility assay with similar results as observed with the 27 kD gamma-zein gene co-suppressed lines (see Example 2). The trait is semi-dominant rather than recessive, that is the 27 kD gamma-zein level in endosperm shows a strong gene-dosage effect. For example, normal corn, pollinated with pollen from this 27 kD gamma-zein gene knock-out line shows at least 30% suppression of 27 kD gamma-zein protein in the heterozygous endosperm. Grain from these crosses showed a significant (6-10% after 4 hours) improvement in digestibility as demonstrated by the *in vitro* digestibility assay.

Example 4: Suppression of 27 kD Gamma-Zein by Over-Expression of High-Sulfur Proteins through Competition for Biosynthetically Available Pools of Sulfur Amino Acids.

Transgenic plants expressing the l8 kD delta-zein protein or the engineered high-lysine, high-sulfur protein hordothionin 12 (US Patent 5,990,389) in the endosperm showed an 80% decrease in gamma-zein protein levels due to limitations of free sulfur-amino acid pools. Seed from these events were tested essentially under the same conditions as seed from gamma-zein gene co-suppressing events (see Example 2) using the *in vitro* digestibility assay in both the presence and absence of disulfide reducing agents. The results obtained were similar to those described in the previous two Examples. The reduced levels of gamma-zein protein had a large positive impact on dry matter digestibility in the absence of DTT. Comparable results were also obtained using hemizygous seed from top-crossed elite inbreds and hybrids with hordothionin 12 corn as the male parent. Maize plants ectopically expressing l8 kD delta-zein protein or hordothionin 12 protein in corn endosperm were both produced using the top-cross technology. Grain from these plants showed the combined traits of improved amino acid composition and improved digestibility (energy availability). For procedures to determine amino acid composition see: Williams, A.P. 1994. Recent developments in amino acid analysis. In: *Amino Acids in Farm Animal Nutrition*; CAB International, Wallingford, U.K., pp.11-36

Example 5: Cloning of a Novel Maize 18 kD Alpha-globulin.

An 18 kD alpha-globulin full-length cDNA (B73 allele) was cloned from a maize endosperm library (mid and late development). Based on EST numbers alpha-globulin transcripts are relatively rare (compared to other seed protein transcripts) and represent approximately 0.1% of the endosperm mRNA during mid development. Different maize inbred lines showed considerable allelism including several SNP's. The 18 kD alpha-globulin gene has been located on chromosome 6, bin 6.05.

The coding region of the B73 allele is 618 bp. The encoded 206 amino acid sequence of the pro-polypeptide contains a predicted N-terminal ER import signal peptide of 23 amino acids. Remarkable is the string of tryptophane (W) residues ("tryptophane box"), which has been also observed in puroindolins from wheat. Puroindolins in wheat have been associated with grain hardness. The 18 kD alpha-globulin of the present invention and the puroindolins are distantly evolutionary related and belong both to the 2S albumin gene superfamily.

<u>SNP's and Alleles</u>

Different corn inbred lines show considerable allelism. For example, sequence fragments isolated from B73 and Mo17 are shown below. The two alleles differ by mostly insertions '*' and a few SNP's (lower case bold). (See also SEQ ID NOS: 9 and 10).

18 kD alpha-globulin, B73 allele, partial
AATTCGCCCTTGTCATTCTGGATTTGCACGCGCACAGTACACATGCTGCG**t**CTTG
CAC**g**TCGCGCCGACTC**g**CT**t**T*********AACC**a**TGGTAGCTAGTACTGGTCGCCGC
CGGAGAAGATGCTGCACTCCTGGGGCTCCGACAGCCGGCACATCATCGGCAAC
CCCGCGGCGTACTCCCGGGCCTTCGTAAGCCTCACGCGGCCGATCCTTGGCCC
GCCGCCGGTGGTGCCGGGACGACACGGTGGATACATCTGC**cgc**TGG**cca**CCCT**g**
aCC**gtag**CCGTATCCCTCTCCTGGCCGGCTGCAGGGGTAGTAGTAGCCCCCCTG
TCCTCCTCCTCCTCCCTGCGGCGGCGGCTGCTGCTGCCGCCCCCATGGCCACC
AGCCTTTCTCCAGcGGCGGCATGGCCTCCTCGTAGCCCCTGACCATGCTCCGG
ATGGCGGCGCAGCGGCACTCGCGGCTCACGTCCTGGAGCTGCTGGCAGCACC
GCATCCGGAGCCCGGTGCCCCACCGGAACGGGCCAACGCCGCCGCCGcCGCC
GCCGCCGGTTAGCTGCCGGTCGAGGAAAGGGCG

18 kD alpha-globulin, Mo17 allele, partial
AATTCGCCCTTGTCATTCTGGATTTGCACGCGCACAGTACACATGCTGCGCCTT
GCACGTCGCGCCGACTCACTCTTTTTTTTTTAACCCTGGTAGCTAGTACTGGTCG
CCGCCGGAGAAGATGCTGCACTCCTGGGGCTCCGACAGCCGGCACATCATCGG
CAACCCCGCGGCGTACTCCCGGGCCTTCGTAAGCCTCACGCGGCCGATCCTTG
GCCC******GGTGGTGCCGGGACGACACGGTGGATACATCTGCGTTTGGTATCCC
TCTCCTGCCC**********GGCTGCAGGGGTAGTAGTAGCCCCCCTGTCCTC
CTCCTCCTCCCTGCGGCGGCGGCTGCTGCTGCCGCCCCCATGGCCACCAGCCT
TTCTCCAGAGGCGGCATGGCCTCCTCGTAGCCCCTGACCATGCTCCGGATGGC
GGCGCAGCGGCACTCGCGGCTCACGTCCTGGAGCTGCTGGCAGCACCGCATC
CGGAGCCCGGTGCCCCACCGGAACGGGCC*********GCCGACGCCGCCGCCGG
TTAGCTGCCGGTCGAGGAAAGGGCG

Example 6: Transgenic Expression of 18 kD Alpha-globulin in Maize.

The cDNA encoding the maize 18 kD alpha-globulin was placed under the control of the strong endosperm specific gamma-zein promoter and introduced into maize plants by *Agrobacterium*-mediated transformation. Several transgenic events were identified that had increased levels alpha-globulin protein as demonstrated by SDS-PAGE and staining of gels with Coomassie blue. A prominent band was visible at a molecular weight corresponding to the 18 kD protein extracted from transgenic seed, but absent from protein extracted similarly from wild type seed. The seed of transformants and progeny overexpressing 18kD alpha-globulin is phenotypically normal (vitreous).

The identity of the polypeptide migrating at 18 kD in the polyacrylamide gel was confirmed by immune blotting using 18 kD alpha-globulin protein specific antibodies. In seed of transgenic plants, the 18 kD alpha-globulin protein accumulates to levels of between 2-5% of the SDS-sample buffer (60 mM Tris, pH 6.8, 100 mM DTT, 2% SDS) extractable seed protein. Seed expressing these amounts of omega zein protein contain 0.12% tryptophan per dry weight compared to No. 2 yellow corn having 0.06% tryptophan amounting to a 100% increase in tryptophan levels. Also, sulfur amino acid content was increased by about 80%. In vitro dry matter digestibility of corn overexpressing 18kD alpha-globulin was determined using the monogastric EDDM assay.

18kD Alpha-globulin overexpression resulted in improved 4 hr EDDM by 10.6 percentage units. An overnight soak in 10 mM of the strong reducing agent

dithiothreietol (DTT), known to maximize in vitro digestibility. Improved digestibility beyond that reached with 18kD alpha-globulin overexpression (by 3.4 percentage units), indicated that the improvement in digestibility attainable with removing digestion-limiting disulfide bonds is partially additive to the improvement obtained with alpha-globulin overexpression. Similarly, improvements made by combining gamma zein co-suppression and 18 kD alpha globulin overexpression can be expected to be partially additive.

Example 7: Preparation of Maize 18 kD Alpha-globulin-Specific Antibodies.

Standard methods for the production of antibodies were used such as those described in Harlow and Lane (1988) *Antibodies: A Laboratory Manual* (Cold Spring Harbor, NY: Cold Spring Harbor Laboratory; incorporated herein in its entirety by reference. Specifically, antibodies for 18 kD alpha-globulin polypeptides were produced by injecting female New Zealand white rabbits (Bethyl Laboratory, Montgomery, Tex.) six times with homogenized polyacrylamide gel slices containing 100 micrograms of PAGE purified alpha-globulin polypeptide. The alpha-globulin polypeptide was purified by sub-cloning into a pET28 vector resulting in an insert encoding a His-tag fusion of the alpha-globulin polypeptide. The fusion protein was expressed in *E. coli* BL21(DE3) cells and purified from the lysate by Nickel chelation chromatography. The denatured purified fusion protein was used for immunization.

Animals were then bled at two week intervals. The antibodies were further purified by affinity-chromatography with Affigel 15 (BioRad)-immobilized antigen as described by Harlow and Lane (1988) *Antibodies: A Laboratory Manual*, Cold Spring Harbor, N.Y. The affinity column was prepared with purified 18 kD alpha-globulin protein essentially as recommended by BioRad RTM. Immune detection of antigens on PVDF blots was carried out following the protocol of Meyer *et al.* (1988) *J. Cell. Biol. 107*:163; incorporated herein in its entirety by reference, using the ECL kit from Amersham (Arlington Heights, Ill.).

Example 8: Cloning of a Maize 50 kD Legumin 1 Prolamin.

A 50 kD legumin 1 nucleotide sequence was cloned from a maize endosperm cDNA library (mid and late development). Based on EST numbers 50 kD legumin 1 transcripts are relatively abundant (compared to other seed protein transcripts) and represent approximately 0.5% of the endosperm mRNA during mid development. The 50 kD legumin 1 DNA sequences isolated from different inbred lines showed a considerable level of polymorphism. The 50 kD legumin 1 gene has been mapped to chromosome 6, Bin 6.01.

Example 9: Preparation of Maize 50 kD Legumin 1-Specific Antibodies.

Antibodies to this protein were prepared essentially as described for the 18 kD alpha-globulin polypeptide.

Example 10: Transgenic Expression of 50 kD Legumin 1 in Maize.

Additional copies of the 50 kD legumin 1 cDNA under control of the strong endosperm specific gamma-zein promoter were introduced into transgenic corn plants. Several maize lines were identified that over-express the 50 kD legumin 1 protein. Over-expression was demonstrated by SDS-PAGE and staining of the gels with Coomassie blue. A prominent band was visible at 50 kD in protein extracted from transgenic seed but absent in protein from wild type seed. The identity of the polypeptide band was confirmed to be the 50 kD legumin 1 protein by immune blotting using the 50 kD legumin 1 protein specific antibodies. In the seed of transgenic maize plants over-expressing the 50 kD legumin 1 protein, this protein accumulates to levels of between 2-5% of the SDS-sample buffer (60 mM Tris, pH 6.8, 100 mM DTT, 2% SDS) extractable seed protein. The seed over-expressing the 50 kD legumin 1 protein showed a normal (vitreous) phenotype. In addition to overexpression of the 50 kD legumin 1, independent transformants were also obtained in which the legumin 1 gene was silenced as evidenced by reduced protein level using immune blotting. These events were also silenced for the 27 kD gamma zein, by apparent promoter-induced silencing. Finally, one event was obtained in which the 27 kD gamma zein was silenced, but the 50 KD legumin 1 clearly

overexpressed as assessed by SDS-PAGE/Coomassie blue staining and immune blotting. Seed from all these events were phenotypically normal (vitreous)

Two segregating events, both silenced for 27 kD gamma zein, but only one overexpressing the corn legumin 1, were evaluated in the monogastric EDDM assay. 50 kD legumin 1 overexpression in low gamma zein background resulted in improved grain digestibility by about 3.2 percentage units.

These results imply not only that overexpression of corn 50 kD legumin1 improves digestibility, but that these improvements are additive to those obtained with gamma zein co-suppression.

Example 11: *Agrobacterium*-Mediated Transformation of Maize.

For *Agrobacterium*-mediated transformation of maize, a nucleotide sequence encoding a protein of the present invention was operably linked to either the 27 kD gamma-zein promoter or the maize 19 kD alpha-zein (cZ19B1) promoter, and the method of Zhao was employed (U.S. Patent No. 5,981,840, and PCT patent publication WO98/32326; the contents of which are hereby incorporated by reference). Briefly, immature embryos were isolated from maize and the embryos contacted with a suspension of *Agrobacterium*, where the bacteria are capable of transferring the nucleotide sequence of interest to at least one cell of at least one of the immature embryos (step 1: the infection step). In this step the immature embryos were immersed in an *Agrobacterium* suspension for the initiation of inoculation. The embryos were co-cultured for a time with the *Agrobacterium* (step 2: the co-cultivation step). The immature embryos were cultured on solid medium following the infection step. Following this co-cultivation period an optional "resting" step is contemplated. In this resting step, the embryos were incubated in the presence of at least one antibiotic known to inhibit the growth of *Agrobacterium* without the addition of a selective agent for plant transformants (step 3: resting step). The immature embryos were cultured on solid medium with antibiotic, but without a selecting agent, for elimination of *Agrobacterium* and for a resting phase for the infected cells. Next, inoculated embryos were cultured on medium containing a selective agent and growing transformed callus was recovered (step 4: the selection step). The immature

embryos were cultured on solid medium with a selective agent resulting in the selective growth of transformed cells. The callus was then regenerated into plants (step 5: the regeneration step), and calli grown on selective medium were cultured on solid medium to regenerate the plants.

Example 12: *Agrobacterium*-Mediated Transformation of Sorghum.

For *Agrobacterium*-mediated transformation of sorghum the method of Cai *et al.* can be employed (US Patent Application No. 09/056,418), the contents of which are hereby incorporated by reference). This method can be employed with a nucleotide sequence encoding any of the proteins of the present invention using the promoters described in Example 11 herein, or another suitable promoter.

Example 13: Transformation of Maize Embryos by Particle Bombardment.

Immature maize embryos from greenhouse donor plants are bombarded with a plasmid containing the nucleotide sequence encoding a protein of the present invention operably linked to a selected promoter plus a plasmid containing the selectable marker gene PAT (Wohlleben *et al.* (1988) *Gene 70*:25-37) that confers resistance to the herbicide Bialaphos. Transformation is performed as follows.

Preparation of Target Tissue

The ears are surface sterilized in 30% Clorox bleach plus 0.5% Micro detergent for 20 minutes, and rinsed two times with sterile water. The immature embryos are excised and placed embryo axis side down (scutellum side up), 25 embryos per plate, on 560Y medium for 4 hours and then aligned within the 2.5-cm target zone in preparation for bombardment.

Preparation of DNA

A plasmid vector comprising the nucleotide sequence encoding a protein of the present invention operably linked to a promoter is made. This plasmid DNA plus plasmid DNA containing a PAT selectable marker is precipitated onto 1.1 μm (average diameter) tungsten pellets using a $CaCl_2$ precipitation procedure as follows:

100 µl prepared tungsten particles in water

10 µl (1 µg) DNA in Tris EDTA buffer (1 µg total)

100 µl 2.5 M $CaC1_2$

10 µl 0.1 M spermidine

Each reagent is added sequentially to the tungsten particle suspension, while maintained on the multitube vortexer. The final mixture is sonicated briefly and allowed to incubate under constant vortexing for 10 minutes. After the precipitation period, the tubes are centrifuged briefly, liquid removed, washed with 500 ml 100% ethanol, and centrifuged for 30 seconds. Again the liquid is removed, and 105 µl 100% ethanol is added to the final tungsten particle pellet. For particle gun bombardment, the tungsten/DNA particles are briefly sonicated and 10 µl spotted onto the center of each macrocarrier and allowed to dry about 2 minutes before bombardment.

<u>Particle Gun Treatment</u>

The sample plates are bombarded at level #4 in particle gun #HE34-1 or #HE34-2. All samples receive a single shot at 650 PSI, with a total of ten aliquots taken from each tube of prepared particles/DNA.

<u>Subsequent Treatment</u>

Following bombardment, the embryos are kept on 560Y medium for 2 days, then transferred to 560R selection medium containing 3 mg/liter Bialaphos, and subcultured every 2 weeks. After approximately 10 weeks of selection, selection-resistant callus clones are transferred to 288J medium to initiate plant regeneration. Following somatic embryo maturation (2-4 weeks), well-developed somatic embryos are transferred to medium for germination and transferred to the lighted culture room. Approximately 7-10 days later, developing plantlets are transferred to 272V hormone-free medium in tubes for 7-10 days until plantlets are well established. Plants are then transferred to inserts in flats (equivalent to 2.5" pot) containing potting soil and grown for 1 week in a growth chamber, subsequently grown an additional 1-2 weeks

in the greenhouse, then transferred to classic 600 pots (1.6 gallon) and grown to maturity. Plants are monitored and scored for the desired phenotypic trait.

Bombardment and Culture Media

Bombardment medium (560Y) comprises 4.0 g/l N6 basal salts (SIGMA C-1416), 1.0 ml/l Eriksson's Vitamin Mix (1000X SIGMA-1511), 0.5 mg/l thiamine HCl, 120.0 g/l sucrose, 1.0 mg/l 2,4-D, and 2.88 g/l L-proline (brought to volume with D-I H_20 following adjustment to pH 5.8 with KOH); 2.0 g/l Gelrite (added after bringing to volume with D-I H_20); and 8.5 mg/l silver nitrate (added after sterilizing the medium and cooling to room temperature). Selection medium (560R) comprises 4.0 g/l N6 basal salts (SIGMA C-1416), 1.0 ml/l Eriksson's Vitamin Mix (1000X SIGMA-1511), 0.5 mg/l thiamine HCl, 30.0 g/l sucrose, and 2.0 mg/l 2,4-D (brought to volume with D-I H_20 following adjustment to pH 5.8 with KOH); 3.0 g/l Gelrite (added after bringing to volume with D-I H_20); and 0.85 mg/l silver nitrate and 3.0 mg/l bialaphos(both added after sterilizing the medium and cooling to room temperature).

Plant regeneration medium (288J) comprises 4.3 g/l MS salts (GIBCO 11117-074), 5.0 ml/l MS vitamins stock solution (0.100 g nicotinic acid, 0.02 g/l thiamine HCL, 0.10 g/l pyridoxine HCL, and 0.40 g/l glycine brought to volume with polished D-I H_20) (Murashige and Skoog (1962) *Physiol. Plant. 15*:473), 100 mg/l myo-inositol, 0.5 mg/l zeatin, 60 g/l sucrose, and 1.0 ml/l of 0.1 mM abscisic acid (brought to volume with polished D-I H_20 after adjusting to pH 5.6); 3.0 g/l Gelrite (added after bringing to volume with D-I H_20); and 1.0 mg/l indoleacetic acid and 3.0 mg/l bialaphos (added after sterilizing the medium and cooling to 60°C). Hormone-free medium (272V) comprises 4.3 g/l MS salts (GIBCO 11117-074), 5.0 ml/l MS vitamins stock solution (0.100 g/l nicotinic acid, 0.02 g/l thiamine HCL, 0.10 g/l pyridoxine HCL, and 0.40 g/l glycine brought to volume with polished D-I H_20), 0.1 g/l myo-inositol, and 40.0 g/l sucrose (brought to volume with polished D-I H_20 after adjusting pH to 5.6); and 6 g/l bacto-agar (added after bringing to volume with polished D-I H_20), sterilized and cooled to 60°C.

Example 16: Transformation of Rice Embryogenic Callus by Bombardment.

Embryogenic callus cultures derived from the scutellum of germinating seeds serve as the source material for transformation experiments. This material is generated by germinating sterile rice seeds on a callus initiation media (MS salts, Nitsch and Nitsch vitamins, 1.0 mg/l 2,4-D and 10 M $AgNO_3$) in the dark at 27-28°C. Embryogenic callus proliferating from the scutellum of the embryos is then transferred to CM media (N6 salts, Nitsch and Nitsch vitamins, 1 mg/1 2,4-D, Chu *et al.*, 1985, *Sci. Sinica 18*:659-668). Callus cultures are maintained on CM by routine sub-culture at two week intervals and used for transformation within 10 weeks of initiation.

Callus is prepared for transformation by subculturing 0.5-1.0 mm pieces approximately 1 mm apart, arranged in a circular area of about 4 cm in diameter, in the center of a circle of Whatman #541 paper placed on CM media. The plates with callus are incubated in the dark at 27-28 C for 3-5 days. Prior to bombardment, the filters with callus are transferred to CM supplemented with 0.25 M mannitol and 0.25 M sorbitol for 3 hr. in the dark. The petri dish lids are then left ajar for 20-45 minutes in a sterile hood to allow moisture on tissue to dissipate.

Circular plasmid DNA from two different plasmids one containing the selectable marker for rice transformation and one containing the nucleotide of the invention, are co-precipitated onto the surface of gold particles. To accomplish this, a total of 10 g of DNA at a 2:1 ratio of trait:selectable marker DNAs is added to a 50 l aliquot of gold particles resuspended at a concentration of 60 mg ml^{-1}. Calcium chloride (50 l of a 2.5 M solution) and spermidine (20 l of a 0.1 M solution) are then added to the gold-DNA suspension as the tube is vortexing for 3 min. The gold particles are centrifuged in a microfuge for 1 sec and the supernatant removed. The gold particles are then washed twice with 1 ml of absolute ethanol and then resuspended in 50 l of absolute ethanol and sonicated (bath sonicator) for one second to disperse the gold particles. The gold suspension is incubated at -70 C for five minutes and sonicated (bath sonicator) if needed to disperse the particles. Six l of the DNA-coated gold particles are then loaded onto mylar macrocarrier disks and the ethanol is allowed to evaporate.

At the end of the drying period, a petri dish containing the tissue is placed in the chamber of the PDS-1000/He. The air in the chamber is then evacuated to a vacuum of 28-29 inches Hg. The macrocarrier is accelerated with a helium shock wave using a rupture membrane that bursts when the He pressure in the shock tube reaches 1080-1100 psi. The tissue is placed approximately 8 cm from the stopping screen and the callus is bombarded two times. Five to seven plates of tissue are bombarded in this way with the DNA-coated gold particles. Following bombardment, the callus tissue is transferred to CM media without supplemental sorbitol or mannitol.

Within 3-5 days after bombardment the callus tissue is transferred to SM media (CM medium containing 50 mg/l hygromycin). To accomplish this, callus tissue is transferred from plates to sterile 50 ml conical tubes and weighed. Molten top-agar at 40°C is added using 2.5 ml of top agar/100 mg of callus. Callus clumps are broken into fragments of less than 2 mm diameter by repeated dispensing through a 10 ml pipet. Three ml aliquots of the callus suspension are plated onto fresh SM media and the plates incubated in the dark for 4 weeks at 27-28°C. After 4 weeks, transgenic callus events are identified, transferred to fresh SM plates and grown for an additional 2 weeks in the dark at 27-28°C.

Growing callus is transferred to RM1 media (MS salts, Nitsch and Nitsch vitamins, 2% sucrose, 3% sorbitol, 0.4% gelrite + 50 ppm hyg B) for 2 weeks in the dark at 25°C. After 2 weeks the callus is transferred to RM2 media (MS salts, Nitsch and Nitsch vitamins, 3% sucrose, 0.4% gelrite + 50 ppm hyg B) and placed under cool white light (~40 $Em^{-2}s^{-1}$) with a 12 hr photoperiod at 25°C and 30-40% humidity. After 2-4 weeks in the light, callus generally begins to organize, and form shoots. Shoots are removed from surrounding callus/media and gently transferred to RM3 media (1/2 x MS salts, Nitsch and Nitsch vitamins, 1% sucrose + 50 ppm hygromycin B) in phytatrays (Sigma Chemical Co., St. Louis, MO) and incubation is continued using the same conditions as described in the previous step.

Plants are transferred from RM3 to 4" pots containing Metro mix 350 after 2-3 weeks, when sufficient root and shoot growth has occurred. Plants are grown using a 12 hr/12 hr light/dark cycle using ~30/18°C day/night temperature regimen.

Example 17: Breeding crosses made with transformed low gamma zein lines

A transgenic line that segregated for co-suppressed 4-coumarate ligase (4CL) was planted and the segregating progeny was either self-fertilized or pollinated with the transgenic low gamma zein line (CS27). Ground grain samples were subjected to a two-stage in vitro-mimicking small intestinal digestion followed by large-intestinal fermentation. The improvement in digestibility for low gamma zein corn in this two-stage assay, and the independent improvement obtained with 4CL co-suppression were additive as indicated by the absence of interaction.

All publications and patent applications mentioned in the specification are indicative of the level of those skilled in the art to which this invention pertains. All publications and patent applications are herein incorporated by reference to the same extent as if each individual publication or patent application was specifically and individually indicated to be incorporated by reference.

Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be obvious that certain changes and modifications may be practiced within the scope of the appended claims.